

Homeostatic maintenance of the murine corneal epithelium in pathophysiological contexts

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Summary

Cornea is the outermost surface of the eye that refracts light to the lens and protects the sensitive ocular machinery. The cornea is divided to three cellular compartments; epithelium, stroma and endothelium. Our work focuses on the corneal epithelium, which is located closest to the tear film and, together with the film, forms a physiological barrier to pathogens and small particles from the environment. We followed the maturation of the mouse corneal epithelium from birth to adulthood and discovered a novel marker, *Krt19*, in this process. *Krt19* expression gradually restricted from the central cornea to the limbus, concomitantly with eyelid opening and epithelial stratification, which are the hallmarks of postnatal maturation of the murine cornea.

Corneal epithelium is renewed continuously throughout life by stem cells. Previous studies demonstrated that the limbus, located in the periphery of the cornea, houses the corneal stem cells. Immediate progeny of the stem cells, the progenitor cells, localize to the limbus, peripheral, and central cornea. We identified the gene *Bmil* in the corneal, epithelial progenitor cells. By lineage tracing of the *Bmil*⁺ cells, we followed renewal dynamics in the central cornea and estimated the turnover of the epithelium to be 2-8 weeks in adult mice. However, we noticed a decrease in renewal rate with older animals. This is in line with evidence from renewal studies of the limbal stem cells, suggesting a general decrease of corneal epithelial renewal upon aging.

We optimized a method to perform *in vivo* epithelial abrasion injury on mouse cornea. The development of this assay was instrumental for the experiments that followed. Using the abrasion model, we showed that the *Bmil*⁺, central, corneal progenitor cells do not contribute to wound healing. Instead, the wound closed by rearrangement and migration of the remaining epithelial cells.

We extended our analysis of the corneal barrier to encompass an accessory organ of the eye, the lacrimal gland (LG, tear gland). LG produces and secretes the aqueous part of the tear film, which is the largest portion of the film. The tear film provides another layer of protection to the ocular surface, because it contains anti-inflammatory and antimicrobial components as well as assists eyelid movements. We studied the role of Ectodysplasin-A (*Eda*) gene in the LG. *Eda* is critical in the development of ectodermal appendages, however LG development was not affected by the loss-of-function mutation in *Eda*. Instead, lack of EDA resulted in modulation of LG secretion and the development of a dry eye disease (DED). Furthermore, we discovered that *Eda* signalling activity was inhibited in response to corneal injury and suggest that this is necessary for the production of reflex tears that are released in ocular insult. In this assay, we shed light on the cooperation between cornea and the LG in homeostasis and injury.

Our work is part of the research that aims to understand maturation and homeostatic maintenance of the anterior segment of the eye, cornea and the LG. This work provides new information regarding the development of *Eda*-linked DED. This is of importance, because the DED affects a large part of the population. Furthermore, we call for further studies on the mechanisms of how these two tissues communicate, as they are intricately linked and dependent of each other.

Table of contents

Summary.....	
List of original publications	i
Abbreviations	ii
1. Introduction.....	1
2. Review of the literature	2
2.1. <i>Stem cells</i>	2
2.1.1. Stem cells in homeostasis.....	2
2.1.2. The stem cell niche	3
2.1.3. Tools to identify stem cells	5
2.1.4. Mammalian cells hold plasticity	8
2.1.5. <i>Bmi1</i>	9
2.2. <i>Cornea</i>	11
2.2.1. Development of the eye	12
2.2.2. Development of the cornea	12
2.2.3. Maturation of the cornea	14
2.2.4. Constructing the stem cell niche	16
2.2.5. Homeostatic renewal of the cornea	17
2.3. <i>Lacrimal gland</i>	20
2.3.1. Development of the LG.....	21
2.3.2. Maturation and maintenance of the LG	22
2.3.3. The connections between cornea and the lacrimal gland.....	23
2.4. <i>Corneal defects</i>	23
2.4.1. Artificially induced corneal injury	24
2.4.2. Ectodysplasin-A in corneal health	25
3. Aims of the study.....	26
4. Material and methods.....	27
5. Results and discussion	29
5.1. <i>General eye growth and establishment of the adult gene expression pattern define corneal maturation (study I)</i>	29
5.2. <i>Bmi1 is expressed in corneal epithelial progenitor cells (study I)</i>	30
5.2.1. Aging slows corneal renewal	32
5.3. <i>Eda is dispensable during LG development, but instrumental in maturation (study III)</i>	33
5.4. <i>Corneal wounding by abrasion is a useful tool to study healing and the cornea-LG connection (studies I, II and III)</i>	35
5.4.1. <i>Bmi1</i> + corneal epithelial progenitor cells do not contribute to corneal wound healing.....	37
5.4.2. Corneal abrasion induces gene expression changes in the LG	37
6. Concluding remarks and future perspectives	39
Acknowledgements	42
References.....	43

List of original publications

- I. **Kalha S**, Shrestha B, Sanz Navarro M, Jones KB, Klein OD, Michon F. (2018). *Bmi1+ Progenitor Cell Dynamics in Murine Cornea During Homeostasis and Wound Healing*. Stem Cells, 36(4), 562-573.
- II. **Kalha S**, Kuony A, Michon F. (2018). *Corneal Epithelial Abrasion with Ocular Burr As a Model for Cornea Wound Healing*. Journal of Visual Experiments, 10(137).
- III. Kuony A, Ikkala K, **Kalha S**, Magalhães AC, Pirttiniemi A, Michon F. (2019) *Ectodysplasin-A signaling is a key integrator in the lacrimal gland-cornea feedback loop*. Development, 146(14).

In addition, unpublished results are presented.

Publications are referred in the text by their roman numerals.

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Abbreviations

BM	basement membrane
Bmi1	BMI1 polycomb ring finger oncogene
Bmp	Bone morphogenetic protein
BrdU	5-bromodeoxyuridine
CBC	crypt base columnar
DED	dry eye disease
E	embryonic day
ECM	extracellular matrix
Eda	Ectodysplasin-A
Edar	Ectodysplasin-A receptor
EGF	epidermal growth factor
EpiSC	epidermal stem cell
EPU	epidermal proliferative unit
Fgf	Fibroblast growth factor
GW	gestational week
HED	hypohidrotic ectodermal dysplasia
HSC	hematopoietic stem cell
iPSC	induced pluripotent stem cell
Krt	keratin
LESC	limbal epithelial stem cell
LG	lacrimal gland
Lgr5	leucine-rich repeat-containing G-protein coupled receptor 5
LRC	label retaining cell
LSCD	limbal stem cell deficiency
mRNA	messenger RNA
MSC	mesenchymal stem cell
NCC	neural crest cell
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
Notch	Translocation-associated Notch protein
P	postnatal day
Pax6	paired box gene 6
PCR	polymerase chain reaction
PRC	polycomb group complex
Shh	Sonic hedgehog
UV	ultraviolet
Wnt	Wingless-type MMTV integration site family member

1. Introduction

The maintenance of organs and tissues in the ever-changing conditions of life presents one of the most interesting questions to biologists. The developmental cells operate in a dynamic, malleable environment of the embryogenesis, whereas during maturation cells localize to their final positions and switch the developmental gene expression network to the adult, homeostatic network. Moreover, cells must be able to respond to injury and orchestrate the complex process of healing that takes place after an insult.

Stem cells function as a key element in these events. In this thesis, I review the knowledge on tissue-specific stem cells in homeostasis and injury. Furthermore, I discuss the functions of the stem cells via their progeny; the progenitors and the differentiated cells.

As a model tissue, I focus on the surface of the eye, the cornea and the lacrimal gland (LG). In the cornea, the tissue-specific stem cells reside in the periphery of the cornea, in the limbus. The limbal, epithelial stem cells (LESC) are slowly-cycling but can proliferate more intensively upon injury. Thus, the cornea provides an example of dynamic changes in the function of the stem cells by external conditions. In addition, I discuss the development and maturation of the cornea and the LG. Lastly, I dissect the mechanisms of how these tissues interact in homeostasis and injury, establishing a platform to study the cooperation of two tissues in the maintenance of vision.

2. Review of the literature

2.1. Stem cells

Our bodies are complex constructs of organs and tissues that maintain the form of an organism. Sensory, nervous, supportive, surface, and muscular tissues assemble into organs that all have their highly specified design and function. The cells within tissues distribute to epithelial, mesenchymal and endothelial categories based on their morphology and location. The epithelium covers all bodily surfaces, such as skin, intestine, and surface of organs, whereas mesenchyme composes of a variety of cell types in the connective tissue. Endothelial cells line the inner surface of blood and lymphatic vessels. Throughout life, cells forfeit in processes of cell loss via desquamation, atrophy, or injury. Dead and desquamated cells are replaced by new cells. The continuous production of emergent cells is fuelled by tissue-specific stem cells, that are able to both self-renew and produce differentiated daughter cells (Lajtha, 1979; Till et al., 1964). Stem cells were first identified in the murine blood-forming tissue and represent the hematopoietic stem cells (HSC) (Becker et al., 1963; Till and McCulloch, 1961). Typically, epithelium is replaced by tissue-specific stem cells that generate new cells only in that tissue. Mesenchymal stem cell (MSC) pool is shared as they produce several different types of cells. Remarkably, MSCs give rise to osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells), and adipocytes (fat cells). The repertoire of stem cells reflects the diversity of organs and tissues.

Stem cells occupy an elemental position in the organism; they are the source of new, differentiated cells in adult tissues. In order to meet the tissue-specific needs, most stem cells produce undifferentiated daughter cells. These cells are commonly referred to as transit-amplifying or progenitor cells, as they are capable of numerous proliferation events, but have a limited life span (transit time) before maturing to fully differentiated cells (Lajtha, 1979).

2.1.1. Stem cells in homeostasis

The proliferation of stem cells to compensate for physiological cell loss is referred to as homeostatic renewal. To maintain tissue homeostasis, stem cells divide either asymmetrically or symmetrically (Fig. 1). In the asymmetric model, a stem cell division gives rise to two daughter cells that are dissimilar: a stem cell and a progenitor cell (Fig. 1A). The stem cell preserves the stem cell population, whereas the progenitor cell departs to differentiation. Divisional asymmetry can arise from a polarization of the stem cell prior to division, such as in *Drosophila melanogaster* (fruit fly) male germ line stem cells (Cheng et al., 2011; Yamashita et al., 2003), or due to cues of the local microenvironment (Voog and Jones, 2010). In this model the stem cell population is maintained invariant over extended periods of time (Klein and Simons, 2011). The symmetric stem cell division produces two identical cells; either two stem cells (Fig. 1 B) or two progenitor cells (Fig. 1 C). Symmetrical division pattern requires special mechanisms to maintain a stable tissue homeostasis.

Early stem cell scientists recognized this issue and postulated that symmetrical divisions are behind HSC maintenance, since stem cell colonies were not equal in size, as is expected from asymmetrically dividing stem cells, but some colonies were larger than others (Till et al., 1964). To explain this, scientists have proposed a neutral drift model (Klein et al., 2010). This model suggests

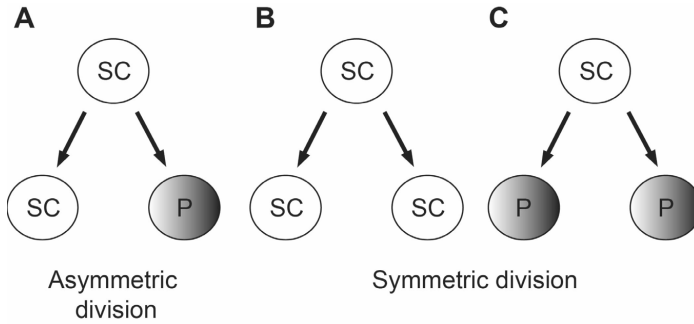


Figure 1: Two models of stem cell division. A stem cell (SC) can follow either an asymmetric or symmetric division model. Asymmetric divisions results in one SC and one progenitor cell (P) that will eventually undergo differentiation. Symmetric divisions generate either two SCs or two Ps.

that individual stem cells are equipotent, but the divisional outcome is stochastic. The following neutral competition between individual stem cells leads to that, with increasing time, a symmetrically dividing population drifts to a situation where individual stem cell colonies become fewer and larger (Klein and Simons, 2011; Klein et al., 2010). Ultimately, stem cell colonies become monoclonal (Snippert et al., 2010). This is also referred to as population asymmetry, because some stem cell colonies diminish and vanish as a result of symmetric divisions to progenitor state, but other colonies are maintained so that the average cell fate outcome in the population level is similar as in the divisional asymmetry model (Klein and Simons, 2011). It is noteworthy, that even in symmetric division the short-term divisional behaviour of stem cells might be predictable for example by marker expression, underlining the fact that the neutral drift model explains a long-term fate of the tissue-specific stem cells as a population rather than their more immediate, short-term outcomes (Nakagawa et al., 2010). Symmetrically dividing stem cells are present in several tissues, including murine testis, interfollicular epidermis (skin) of the ear and back, intestinal epithelium, and most likely in the cornea (Clayton et al., 2007; Doupé et al., 2010; Klein et al., 2010; Lopez-Garcia et al., 2010; Mort et al., 2009; Snippert et al., 2010). Neutral drift dynamics can occur at the level of the progenitor cells as well (Andersen et al., 2019). It is tempting to speculate whether neutral drift is a universal model for stem cell cycling. In addition, the age-dependent decline of stem cell numbers supports the population asymmetry theory (Barrandon and Green, 1987; Dorà et al., 2015).

The mode and rate of stem cell divisions might not be as carved in stone as previously thought. Divisional model depends on the tissue in question and even on the prevailing status; it is possible that during homeostatic renewal a stem cells undergoes asymmetric divisions, but after an injury a symmetrical division pattern takes over to compensate for excessive cell loss.

2.1.2. The stem cell niche

Stem cells reside in tissues in small numbers. For example, in the bone marrow, there is estimated to be only one colony-forming cell (i.e. stem cell) per 10 000 other cells (Till et al., 1964). Each organ and tissue keep their own, tissue-specific stem cells in a location within that tissue. This location is called the stem cell niche and often it represents a morphologically distinguishable place, however not in every case. A multitude of stem cell niches in a wide variety of tissues have been identified. The textbook examples of mammalian stem cell niches include i) the bone marrow niche, where the HSCs reside, ii) the hair follicle bulge and, iii) the intestinal crypt (Barker et al., 2007; Cotsarelis et al., 1990; Kiel et al., 2005; Sangiorgi and Capecchi, 2008; Tumbar et al., 2004). Other well-known

stem cell niches include the interfollicular epidermis that hosts epidermal stem cells (EpiSC), testis with sperm stem cells, spermatogonia, along the seminiferous tubules, and distinct regions of the brain (Clayton et al., 2007; Gonzalez-Perez, 2012; Tegelenbosch and De Rooij, 1993). In addition to these mammalian examples, also *Drosophila* and *Caenorhabditis elegans* (nematode) provide elegant tools for the study of stem cell niches.

The essential function of the stem cell niche is to provide a local, protected microenvironment that maintains the stem cells in an undifferentiated state, but also displays an avenue for differentiation. This task is managed by a multitude of factors from the cellular and extracellular microenvironment. Contact with supporting cells of the niche is an important cellular factor of the local niche environment. In the *Drosophila* male gonad, physical contact with a hub cell is required for stem cell self-renewal and maintenance (Voog et al., 2008). In a murine analogy, spermatogonia are embedded in between Sertoli cells that transmit the hormonal cues from the vasculature and control spermatogenesis (Griswold, 1998). Furthermore, the Paneth cells of the small intestine crypt act as niche supporting cells and secrete WNT to maintain the crypt base columnar (CBC) cells, that are a subpopulation of the intestinal stem cells (Sato et al., 2011b).

In addition to the supporting cells, the extracellular matrix (ECM) functions as an essential niche component. It displays secreted factors as well as adhesive and structural proteins to the stem cells. It is known that factors like WNTs, NOTCHs, BMPs, SHH, FGFs, and more, signal to the niche and maintain the stem cells (Voog and Jones, 2010). The basement membrane (BM) is a part of the ECM that is particularly important for the stem cell niche. For example, the EpiSCs sit in contact with the BM, but lose stem cell identity upon delamination from the membrane. The composition of laminins in the BM together with ECM-cell connections regulate stem cell maintenance in epidermis, hair follicles and mammary gland (Levy et al., 2000; Morgner et al., 2015; Romagnoli et al., 2019). Together the stem cells, supporting cells and the extracellular environment with its intricate signalling circuits constitute the stem cell niche.

Other components have also been introduced in relation to the stem cell niche. Physical factors, such as tissue elasticity can function as a niche component. Biomechanical signalling was shown to regulate EpiSC self-renewal and differentiation (Miroshnikova et al., 2018). On another note, HSCs and spermatogonia preferably reside in close contact to vasculature and possibly obtain some of the niche factors via the bloodstream (Sugiyama et al., 2006; Yoshida et al., 2007). This is an interesting notion and future research will show whether circulating, systemic factors provide a mechanism to coordinate stem cell activities of different tissues in response to whole-organismal changes, such as aging. All in all, the local microenvironment of a stem cell is a three-dimensional composition of a multitude of factors.

Implicit in the stem cell niche model is that when a stem cell exits the niche, it will inevitably lose its stem cell identity and undergo differentiation. This is typically visualized with a positional hierarchy, where stem cells reside in a specific compartment in the niche, and each proliferation event takes the progenitor cells further away from that compartment. Thus, the loss of contact to the niche microenvironment acts as a differentiating factor. This idea has been challenged by research done on the intestinal crypt. Interestingly, the position of a stem cell in the intestinal crypt does not necessarily define its fate (Takeda et al., 2011). Furthermore, research on organoids, small and simple versions of an organ grown *in vitro*, indicates that stem cell self-renewal and differentiation function properly without the physical niche environment as well (Sato et al., 2009). These results question the importance of the stem cell niche in some systems. Interestingly, the van Rhee and Clevers groups

showed that cells from the intestinal progenitor region can return to the crypt and become stem cells again (Ritsma et al., 2014; Van Es et al., 2012). Similarly, progenitors in *Drosophila* ovaries and testis can revert back to germline stem cells upon re-entry to the niche (Brawley and Matunis, 2004; Kai and Spradling, 2004). In conclusion, it seems that the niche has differential significance to its stem cells in different tissues and contexts.

How is the stem cell niche first established? In the developing intestine and sebaceous gland, equipotent, developmental progenitors scatter within the tissue, maintain it during development and follow independent fate choices until the end of morphogenesis, when they restrict to follow the typical renewal pattern of that tissue (Andersen et al., 2019; Guiu et al., 2019). This suggests that stem cells are specified *in situ* in the niche. The timing of initiation of the homeostatic renewal varies between tissues. Importantly, after the stem cell niche is established, it maintains the stem cells in the varying conditions of development, maturation, disease, and aging.

2.1.3. Tools to identify stem cells

In order to better understand stem cells, they need to be identified *in vivo* and *in vitro*. However, stem cells within tissues are rare. Furthermore, they localize to niches, where they reside among other cell types and need to be segregated from them. One of the first *in vivo* methods to locate stem cells is based on their cell cycle dynamics. Characteristically, stem cells divide rarely and can be identified using label retainment, making them so-called label retaining cells (LRC). In a label retainment assay, cells incorporate a nucleoside (thymidine) analog, 5-bromodeoxyuridine (BrdU), 5-ethynyl-2'-deoxyuridine (EdU) or tritiated thymidine, in their DNA during cell division. All cells that divide often dilute and eventually lose the label, but label retains in cells that divide infrequently. Using BrdU, EdU or tritiated thymidine, LRCs have been identified in many tissues, including the hair follicle, intestine, murine incisor, epidermis, dental, and olfactory epithelia (Bickenbach and Mackenzie, 1984; Cotsarelis et al., 1990; Harada et al., 1999; Jang et al., 2014; Morris and Potten, 1994; Potten et al., 1974). Label retainment was a critical method for the first discovery of tissue-specific stem cells and their niches. Thus, especially in tissues where the stem cells have not been identified before, the localization of LRCs initiates the study of stem cells. In addition to BrdU, EdU and tritiated thymidine, other methods for label retainment exist as well. Tumbar and coworkers developed an approach which utilizes transgenic mice with a fluorescent label in histone 2B protein to track the LRCs in skin (Tumbar et al., 2004). However, not all stem cells are slowly-cycling and cannot be identified with a label retainment assay (Fuchs, 2009).

Perhaps the most intuitive approach to locate a stem cell *in vivo* is by detecting the expression of a gene. A gene that is specifically expressed in a given cell is called a marker gene. Unfortunately, and maybe counterintuitively, a uniform marker for all stem cells does not exist. It is more appropriate to try to find a tissue-specific stem cell marker, however most stem cells cannot be identified with that kind of marker gene either. A putative gene is either only expressed in a subpopulation of stem cells or encompasses a larger expression domain. Still, some useful markers exist. HSCs express many feasible markers, such as *c-kit*, *Sca-1*, and members of the CD (cluster of differentiation) gene family (Garg et al., 2013; Wognum et al., 2003). The hallmark of intestinal CBC cells is the expression of *Lgr5*, whereas the second population of intestinal stem cells, located higher in the crypt, specifically express *Bmi1* (Barker et al., 2007; Sangiorgi and Capecchi, 2008). Interestingly, *Lgr5* is also expressed by stem cells in the bulge region of the hair follicle (Jaks et al., 2008). There is a

multitude of different stem cell markers. More likely than using one marker, in the future tissue-specific stem cells could be identified based on an array of genes that are expressed in them. However, marker expression alone is not reliable enough; it should be combined with another method.

Lineage tracing is a way to identify a cell and its progeny (i.e. the lineage). Lineage tracing provides a revolutionary tool for developmental biology, and excellently works for stem cell biology too. In lineage tracing, the cell's cycling dynamics do not matter, instead the method focuses on the hierarchy of cells. In this method, a cell is permanently marked with a dye or a genetic label. When the marked cell divides, all its daughter cells have the label and can be detected in the tissue. In order to be successful and reliable, lineage tracing must fulfil these criteria (modified from (Hsu, 2015)):

1. A careful assessment of the cells that are marked at the initial time point, so that the starting population(s) is clearly defined.
2. The markers used to label the cells remain exclusively in the original cells and their progeny and will not diffuse to the neighbouring cells.
3. These markers are sufficiently stable and are not toxic to the cells during the entire tracing period.

The most commonly used method for genetic labelling *in vivo* makes use of a site-specific, Cre-mediated recombination of loxP loci (Orban et al., 1992). The *Cre* gene, originally from P1 phage, transcribes a recombinase under a chosen promoter region. The correct choice of a promoter is instrumental; it allows for tissue or cell-type specific expression, or if needed, a general expression of *Cre*. This is also the problem of genetic lineage tracing. A suitable marker gene must be identified in order to choose the promoter correctly. Once this is done, a Cre mouse line is crossed to a mouse line containing a fluorescent label or a pigment-producing enzyme (like β -galactosidase). This is referred to as the reporter. The reporter is inserted into a ubiquitously expressed locus, such as under *Rosa26* promoter, and contains a STOP-cassette, that is flanked by loxP sites on both ends. The STOP-cassette prevents the expression of the reporter. Expression of the reporter is achieved when Cre recombinase excises the loxP flanked STOP-cassette, thus allowing for the expression of the reporter (Fig. 2 A). The removal of the STOP-cassette is permanent, meaning that the labelled cell and all its progeny express the reporter gene. In order to allow for dynamic expression, researchers have generated inducible Cre lines. Here, *Cre* gene is fused with a mutated form of oestrogen receptor (Danielian et al., 1998). This way Cre recombinase functions only in the presence of tamoxifen, the sole ligand of the mutated receptor (Fig. 2 B). Inducible Cre allows for a temporal control of labelling; only cells marked during tamoxifen induction express the reporter and the expression is continued in the progeny of those initially labelled cells. This is especially useful when trying to identify stem and progenitor cells (Fig. 2 C). It is noteworthy that there is a wide variety of lineage tracing strategies and the choice between approaches might make a critical difference in the outcome. Thus, planning, controlling and correctly analysing lineage tracing experiments is important.

In vitro methods are used in parallel with *in vivo* assays for stem cell identification. Colony formation shows the growth potential of a single cell through clonal expansion (Barrandon and Green, 1987). Cell types are identified based on clonal characteristics. Simplistically, paraclones have fully differentiated cells, meroclones contain a mix of differentiated and naïve cells, and holoclones

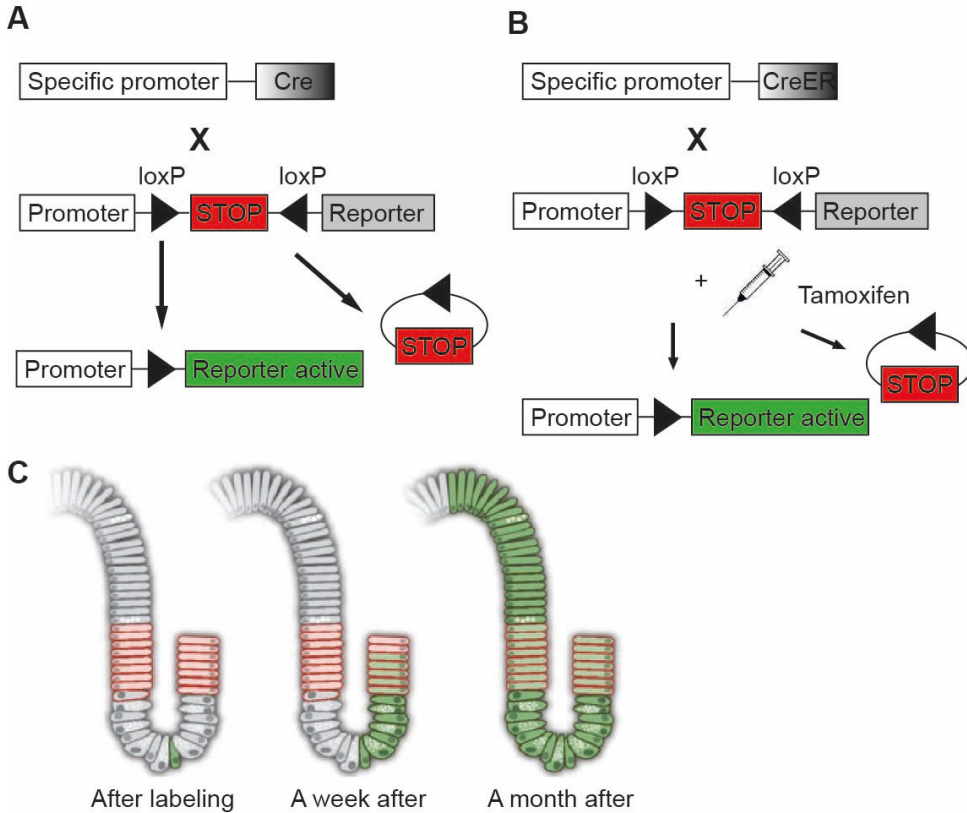


Figure 2: Lineage tracing using the Cre-loxP system. (A) Cre recombinase is expressed under a tissue-specific and/or temporally specific promoter. When combined with an animal with the reporter construct, Cre excises the loxP flanked STOP-cassette and removes it from the construct. This activates reporter expression. (B) Cre is fused with an oestrogen receptor and forms a CreER complex. Once Cre is expressed, only the administration of tamoxifen enables Cre entry to the nucleus and subsequent activation of the reporter. (C) Cre-loxP system is useful to detect a stem cell and its progeny. An individual stem cell is labelled by reporter activation (in green), but the reporter stays active in the progeny of that cell. Progenitor cells are marked with red. Long-term follow-up reveals cellular hierarchy in the small intestine. C modified from (Blanpain and Fuchs, 2014).

encompass the stem cells. Other, commonly used *in vitro* methods are spheroid and organoid assays. Similarly to colony formation, spheroid formation is a 2-dimensional assay, but stem cells produce large clones that obtain a spheroid shape and can be analysed for example immunohistologically. Stem cells in the central nervous system were identified using a spheroid assay (Reynolds and Weiss, 1992). The remarkable *in vitro* potential of stem cells was evidenced by organoid formation (Sato et al., 2009). Here, single *Lgr5*⁺ intestinal stem cells formed a small version of the intestinal crypt in a 3-dimensional *in vitro* environment. In addition to the intestinal organoids, adult stem cells of the stomach, colon, liver, pancreas, and prostate can produce organoids (Bartfeld et al., 2015; Boj et al., 2015; Huch et al., 2015; Karthaus et al., 2014; Sato et al., 2011a).

Together label retainment, stem cell markers, lineage tracing and *in vitro* assays provide a proficient toolkit for a stem cell scientist. Another important method for stem cell identification is transplantation. In fact, using genetic markers originates from early transplantation studies on chicken embryos (Rawles, 1948). Transplantation was also seminal in the identification of HSCs (Becker et al., 1963). New strategies are introduced still today, because a unifying tool to identify stem cells does not exist. It remains to be seen how genomic, epigenomic and transcriptomic approaches will affect stem cell characterization. More than using one tool, stem cell identification relies on a few assays to test whether the studied cells fulfil the stem cell criteria.

2.1.4. Mammalian cells hold plasticity

Homeostatic renewal is delineated as unidirectional; once a stem cell has left the niche and started differentiation, it cannot become a stem cell again. The progressive restriction of (developmental) potential is illustrated in Conrad Waddington's epigenetic landscape (Fig. 3) (Waddington, 1957). Modern science has challenged Waddington's idea, because in some instances differentiated cells can change differentiation state or even cell fate through dedifferentiation or transdifferentiation. In dedifferentiation, a terminally differentiated cell reverts back to a less-differentiated stage from within its own lineage (Jopling et al., 2011). It can then redifferentiate back to the pre-existing tissue. Unlike dedifferentiation, the process of transdifferentiation occurs through conversion of existing cells to a new identity (Jopling et al., 2011; Selman and Kafatos, 1974). Both processes appear after an injury, but they can be artificially induced as well. Most naturally occurring de- and transdifferentiation takes place in non-mammalian species, classical examples include amphibian limb and zebrafish heart regeneration (Jopling et al., 2010; Kikuchi et al., 2010; Tanaka and Reddien, 2011). In general, mammals do not regenerate full appendages or organs.



Figure 3: Waddington's epigenetic landscape. During differentiation, a cell becomes progressively more restricted, as visualized with a ball that rolls down the hill to a basin that reflects the developmental fate or outcome. Dedifferentiation lifts the ball from the basin to a less differentiated platform, from where it will roll back the same developmental path. In transdifferentiation, fate change leads the cells to another fate. (Waddington, 1957).

However, mammalian cells exhibit surprising plasticity that is revealed upon injury or in an experimental setting (Blanpain and Fuchs, 2014; Chang-Panesso and Humphreys, 2017; Tata and Rajagopal, 2017). There are many molecular and physiological routes how the cells undergo regenerative processes (Su, 2018). For example, if parts of the liver are lost, remaining liver cells, hepatocytes, regenerate the lost tissue (Schaub et al., 2014; Yanger et al., 2014). Importantly, liver regeneration occurs without tissue-specific stem cells or transdifferentiation from another cell source.

In large skin wounds the epidermis loses its own EpiSCs from the wounded area, but stem cells from the nearby, unwounded hair follicles can regenerate the epidermis (Ito et al., 2007). Interestingly, regeneration works the other way around too. When the hair follicle bulge, that contains the stem cells, is experimentally ablated, epithelial cells from outside the bulge stem cell niche migrate and proliferate to re-establish the hair follicle stem cell population (Rompolas et al., 2013). In both cases, the regenerative epithelial cells assume a new cell identity, however in the case of epidermal regeneration the identity switch is transient and lasts only until healing is finished.

Furthermore, tissue recombination experiments show that mammalian tissues have potential for remarkable cell fate changes. Here, the epithelial and mesenchymal components of a tissue are enzymatically separated from each other and then combined with an epithelium or mesenchyme from another tissue. In one of the early experiments, rat embryonic epidermis combined with mouse mammary mesenchyme generated the mammary epithelial cell types as well as ductal structures (Cunha et al., 1995). Later, a series of recombinations between adult corneal epithelium and embryonic dermis (the mesenchymal tissue of skin) revealed the stepwise gene expression change that show first a loss of corneal identity, then an intermediate, naïve state followed by differentiation to epidermis (Ferraris et al., 2000; Pearton et al., 2004; Pearton et al., 2005). The plasticity that is revealed upon these tissue recombination experiments is rooted in underlying developmental gene expression programs, since transdifferentiation by recombination requires an embryonic party. All in all, these results show that even in mammals, the resident cell populations adjust fate decisions after wounding or ablation and suggest that cells are molecularly competent to interpret signals that orchestrate the appropriate regeneration processes.

A very special example of cellular plasticity is the generation of induced pluripotent stem cells (iPSC). iPSCs were first made from both embryonic and adult murine fibroblast cells by introducing just four genes (*Sox2*, *Oct3/4*, *c-Myc* and *Klf4*) to them via retroviral transduction (Takahashi and Yamanaka, 2006). iPSCs resemble the pluripotent embryonic stem cells that reside in the inner cell mass of a blastocyst stage embryo. This means that the iPSCs can give rise to all three embryonic germ layers and that they can be maintained indefinitely in culture. Later, iPSCs were made from human fibroblasts as well (Takahashi et al., 2007). iPSCs can be differentiated into a new fate using a directive set of differentiation protocols and this remarkable property opens the possibility of using the iPSCs in regenerative medicine.

2.1.5. *Bmi1*

Bmi1 (also known as PCGF4 and RNF51) belongs to the Polycomb Complex gene family and is situated on chromosome 10. The polycomb group proteins are a major component of the epigenetic regulation machinery of gene expression; they form protein complexes that silence target genes through chromatin remodelling and/or modification of histones. This transcriptional repression is of substantial importance, because it maintains adequate levels of gene expression during development and in adulthood. Polycomb proteins are classified into two functional groups: polycomb group complex (PRC) 1 and 2. BMI1 is an essential member of PRC1 together with CBX4, PH1 and RING1B (Simon and Kingston, 2009). BMI1 and RING1B function in concert to ubiquitylate histone 2A and most studies suggest that this is the key silencing mechanism of the PRC1 complex (Cao et al., 2005; Li et al., 2006). However, histone 2A ubiquitylation does not prevent the access of the transcription complex to the target gene, thus the exact mode of gene silencing is unclear (Breiling et

al., 2001). PRC1 silences hundreds of genes, including developmentally important *Hox*-genes, however special focus has been placed on the repression of the locus *Ink4a/Arf* (Jacobs et al., 1999). This locus transcribes two cell cycle inhibitors, p16^{Ink4a} and p19^{Arf}. These inhibitors repress important cell cycle activators, cyclins, thus contributing to cell cycle arrest. Whereas PRC1 repression of the *Ink4a/Arf* locus could lead to an increase in cell proliferation. However, in addition to the functions of the PRC1, the regulation of cell cycle entails a multitude of other, positive and negative, effectors as well (Poon, 2016).

The role of *Bmi1* has been studied in depth in the context of stem cells and homeostatic renewal. *Bmi1*⁺ stem cells have been identified at least in the hematopoietic system, intestine, central nervous system, murine tooth, and sweat glands (Biehs et al., 2013; Kim et al., 2015; Molofsky et al., 2003; Ohe et al., 2015; Park et al., 2003; Sangiorgi and Capecchi, 2008). In these tissues, *Bmi1* functions in stem cell self-renewal and maintenance, presumably via repression of the *Ink4a/Arf* locus (Biehs et al., 2013; Chatoo et al., 2010; López-Arribillaga et al., 2014). In addition, *Bmi1*⁺ intestinal stem cells show remarkable chemoresistance and proliferative potential as they can replenish the *Lgr5*⁺ intestinal stem cells upon injury, serving as a pool of reserve stem cells in the intestine (Tian et al., 2011; Yan et al., 2012; Zhu et al., 2013). A similar phenomenon occurs in sweat glands as well. *Bmi1*⁺ stem cells resist radiation-injury and replace other sweat gland stem cells quickly after injury (Ohe et al., 2015). In the eye, *Bmi1* maintains the developmental progenitor cells of the retina, thus contributing to normal retinal development and postnatal maturation (Barabino et al., 2016; Chatoo et al., 2010).

Bmi1 maintains a plethora of other functions as well. Loss of PRC1 complex partner RING1B results in the arrest of development at gastrulation (Voncken et al., 2003). Furthermore, PRC1 and 2 maintain X chromosome inactivation and paternally imprinted gene expression pattern (Simon and Kingston, 2009). *Bmi1* expression is necessary for maintaining the redox balance in mitochondria, as loss of *Bmi1* results in an accumulation of reactive oxygen species and activation of the DNA damage response (Liu et al., 2009). Furthermore, *Bmi1* is an oncogene, meaning that aberrant expression of *Bmi1* is associated with cancer (Lessard and Sauvageau, 2003). Mice deficient in *Bmi1* have a number of defects including, malformation of the posterior body parts, severe neurological abnormalities, alterations in hematopoietic cell lineages, and overall shortened lifespan (van der Lugt et al., 1994). Previous studies have documented that approximately half of the *Bmi1*-knockout mice die before weaning and the remaining animals succumb latest by 20 weeks of age (López-Arribillaga et al., 2014; van der Lugt et al., 1994). All in all, BMI1 serves a vital role in the maintenance of an appropriate gene expression level and as a result of that, appears important in a multitude of biological processes, including development and tissue homeostasis.

2.2. Cornea

Cornea is the transparent surface of the eye that protects the sensitive ocular machinery (Fig. 4 A). It distributes this task with the other, superficial parts of the eye; the eyelids, tear fluid, and conjunctiva, the white coverage of the eye that is continuous with the cornea. The cornea is a thin, but extremely durable layer that prevents pathogens and small particles, such as dust, from entering the eye. The cornea also filters harmful ultraviolet radiation (UV). Evidently, the cornea absorbs 34 % of UV-A and 80 % of UV-B radiation that hits the ocular surface, the rest is absorbed by the other parts of the anterior eye (Cejka and Cejkova, 2015). In addition to the protective role, the cornea has optical properties that are instrumental for accurate sight. The cornea refracts light rays that travel through it, thus participating in the processing of visible light information.

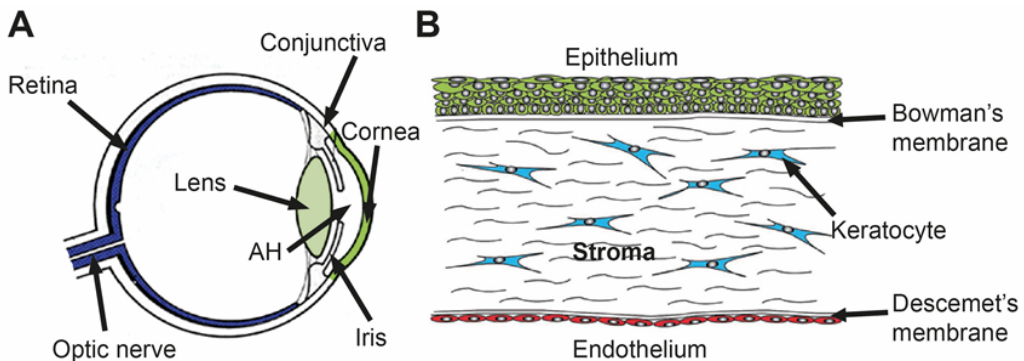


Figure 4: The eye and the cornea. (A) Cornea is the outermost surface of the eye that, together with conjunctiva, covers the lens and the retina. (B) Cornea houses three cellular layers; epithelium, stroma, and endothelium, and two basement membranes. Stromal keratocytes are sparsely distributed in a network of collagen fibers. AH: aqueous humor.

The simple yet highly functional structure of the cornea enables it to operate in its many roles successfully. Cornea is a non-keratinized, thin (0.5 μm in human) and layered tissue (Fig. 4 B). The topmost layer is a stratified squamous epithelium, 4-6 cell layers thick, that rests on a BM, called the Bowman's membrane. The cells that are closest to the BM are basal cells. They are round or square-shaped. They are covered with 2-3 layers of suprabasal cells that have a slightly flattened morphology. Topmost layers are composed of superficial cells that are fully flattened and will be desquamated from the ocular surface to the tear fluid. The epithelial cells are strictly adherent to each other and connected via tight junctions (Sugrue and Zieske, 1997; Wang et al., 1993; Yi et al., 2000; Zhou et al., 2016). This organization makes the corneal epithelium an excellent barrier to the external environment.

Below the Bowman's membrane is the corneal stroma, where the stromal cells, keratocytes, are sparsely located between fibers of collagen. This collagen is mostly of type I, but also collagens V and VI (Robert et al., 2001). Together they make up a lattice arrangement and this way give the cornea its transparency and refractory properties. In fact, stroma is the largest layer of the cornea and maintaining corneal transparency comes down to the stroma. The free passage of light is ensured by lack of blood and lymphatic vessels, avascularity, in the stroma. Antiangiogenic proteins, such as

pigment epithelium–derived factor, angiostatin, restin, and endostatin serve as a barrier to immune infiltration and subsequent vasculature outgrowth in the corneal stroma (Azar, 2006; Qazi et al., 2010).

The stroma is underlined by another BM, the Descemet's membrane. The corneal endothelium is a monolayer of cells that faces the anterior chamber of the eye. The endothelial cells regulate water and solute uptake to the cornea and maintain corneal transparency this way. Corneal nerves innervate all layers of the cornea and thus make it an extremely sensitive tissue.

2.2.1. Development of the eye

The form of an organism arises during embryonic development. The sophisticated architecture of organs and tissues takes shape in a process called morphogenesis, which encompasses a variety of developmental mechanisms. The detailed sequence of developmental events that direct organ development is termed organogenesis. Organogenesis of an eye is a classical and well-studied model of embryonic organ development. Eye development is characterized by inductive interactions, where molecular signalling from one tissue initiates the development of another tissue. Early during embryonic development, the gastrula stage embryo of triploblastic animals separates into three germ layers that define the positions of future organs in an organism. The eye, skin epithelium and the entire nervous system develop from the outermost germ layer, the ectoderm. The eye-forming region of the ectoderm is determined to its fate together with other sensory organs in the preplacodal region of the neural plate or neurula stage embryo, which occurs immediately after gastrula stage (Streit, 2007). At this point, the sensory organs share a common developmental base, but diversify and become competent to respond to different inductive instructions during the next developmental stage.

The first wave of eye development is defined by patterning of the general structures of the eye starting from embryonic day (E) 8 in mouse (during 4th gestational week, GW, in human) (Freund et al., 1996). Liquid-filled vesicles of the forebrain bulge towards the surface ectoderm on both sides of the embryonic head region and form optic vesicles (Hilfer and Yang, 1980). An inductive signal from the optic vesicle to the surface ectoderm initiates the formation of a lens placode, a local thickening of the surface ectoderm just above the optic vesicle. The eye-forming ectoderm must express *Pax6*, a master regulator gene of eye development, otherwise the induction fails and eye development halts or is perturbed (Glaser et al., 1992; Quiring et al., 1994). The lens placode is pulled, i.e. invaginates, into the optic vesicles by adhesive filopodia and induces the optic vesicle to fold in on itself, forming a double-layered optic cup (Chauhan et al., 2009; Hilfer and Yang, 1980). Furthermore, the epithelial cells of the lens placode undergo apical constriction during invagination, where the outer side of the epithelial cells contracts forming a wedge in the epithelial sheet (Plageman et al., 2010; Plageman et al., 2011). The optic cup will later differentiate into neural retina and the pigmented epithelium that covers the retina. The lens placode separates from the surface ectoderm and is fully internalized as it becomes the lens vesicle. The first wave of eye development is finished at E10.5 in mouse and during the 5th GW in human embryos. The second wave extends to the postnatal time period as the cell types of the retina, lens and other parts of the eye specify and mature to their final functional role.

2.2.2. Development of the cornea

The cornea develops from the surface ectoderm together with the other tissues of the anterior eye; the lens, conjunctival epithelium, LGs and eyelid epithelium. During the initial phase of eye

development, the ectodermal eye precursor cells display *Pax6* expression in the preplacodal region (Fig. 5) (Bailey et al., 2006; Bhattacharyya et al., 2004). It is hypothesized, however not experimentally confirmed, that cornea and lens share a common origin. This idea is supported by both physiological and evolutionary evidence and it is referred to as the refraction hypothesis, as the cornea and lens carry out light refraction together, often as one unit (Jonasova and Kozmik, 2008; Piatigorsky, 2001).

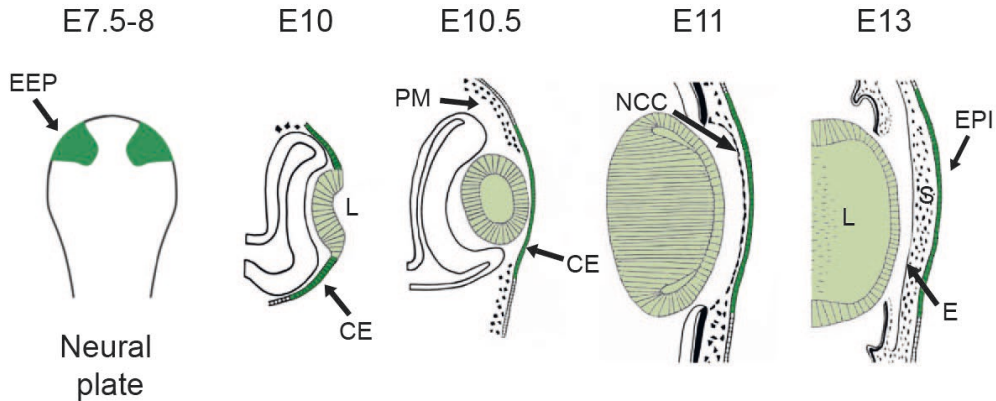


Figure 5: Development of the cornea. Ectodermal eye precursors (EEP) emerge in the preplacodal region of the embryonic neural plate at E7.5-8. Cornea and lens (L) derive from the EEPs that localize to the surface ectoderm. Corneal epithelium develops from the peripheral ectoderm that surrounds the lens and is referred to as corneal ectoderm (CE). At E10.5, CE covers the invaginated lens vesicle. Perioptic mesenchyme (PM) houses the neural crest cells (NCC) that migrate under the CE to form endothelium and stroma at E11. All layers are established by E13. Timeline refers to embryonic development of mouse. Modified from (Collomb et al., 2013).

The primordial lens and corneal tissues separate, when the lens placode specifies and starts to invaginate into the optic cup at E10 (in mouse), leaving the eye-forming ectoderm in the lens placode periphery to establish a corneal fate. The development of the lens is important for the correct development of the cornea (Collomb et al., 2013). However, removal of the lens during eye development results in the absence of cornea only in some cases, but when the cornea develops, it is nevertheless aberrant (Collomb et al., 2013). In line with that, evidence from human patients with absence of lens, aphakia, show that a deformed cornea develops in absence of the lens (Valleix et al., 2006). Further, lens development is crucial for the general growth of the eyeball and the removal of lens affects the morphology of multiple eye regions, such as the retina (Collomb et al., 2013; Hyer et al., 2003). In addition, the lens prevents premature cornea development via Semaphorin 3A signalling (Lwigale and Bronner-Fraser, 2009).

Following lens invagination, the remaining ectodermal eye precursors define as primary corneal epithelium. The developing epithelium comprises only two cell layers and cells appear particularly flattened and immature. Regardless, they start to synthesize collagens between the ectoderm and the lens vesicle, shaping an acellular, primary corneal stroma (Young et al., 2019). This acellular stroma is composed of collagens II, VII and IX (Robert et al., 2001). The formation of the stroma coincides with the stabilization of *Pax6* expression in the corneal epithelium in chick (Collomb et al., 2013). In fact, the sustained expression of *Pax6* is necessary for cornea development and postnatal

maintenance in other species as well (Li et al., 2015; Ouyang et al., 2014; Park et al., 2018). Next, cells from the neural crest (NCC), also an ectoderm derivative, migrate from the perioptic mesenchyme, a temporary tissue surrounding the eye, under the corneal epithelium to constitute the cellular stroma and endothelium. This migration occurs in one or two waves, depending on the species, as reviewed by Dhouailly and colleagues (Dhouailly et al., 2014). In chick, the primary endothelial cells arrive first at E4 and the primary, stromal keratocytes invade the collagen-rich area between the endothelium and the epithelium later, at E6 (Lwigale et al., 2005). In mouse, the migration of the NCCs is completed in one wave that takes place at E11-13 (Pei and Rhodin, 1970). At this point, the stroma resembles an undifferentiated tissue.

The corneal epithelium induces and regulates the molecular environment of the developing stroma and endothelium. Corneal epithelial cells synthesize retinoic acid, which in turn regulates the expression of essential transcription factors, *Foxc1* and *Pitx2*, in the specification of the NCCs to corneal stroma and endothelium (Matt et al., 2005). In the absence of retinoic acid, NCCs fail to initiate this, instead they form a thick mesenchyme-looking tissue that replaces the cornea (Matt et al., 2005). Importantly, *Pitx2* activates *Dkk2* that represses canonical WNT signalling during cornea development (Gage et al., 2014; Kumar and Duester, 2010). Hence, the migratory movements of the NCCs define the three layers of the cornea.

The migratory period finishes the gross morphological development of the cornea. This is followed by formation of the two BMs; Bowman's and Descemet's membranes (Pei and Rhodin, 1970). Membrane development proceeds to generation of anchoring fibrils and hemidesmosomes in the corneal epithelium (Tisdale et al., 1988). The build-up of the anterior chamber fluid, which holds ocular pressure stable, is required for maintenance of the accurate curvature in the cornea. During the last perinatal stages of corneal development, the corneal-type gene expression patterns arise, and cell differentiation begins.

2.2.3. Maturation of the cornea

Postnatal corneal development is often referred to as maturation. However, the concepts of development and maturation are more of a continuum of morphogenetic events that span over the entire lifetime. Some of the maturation processes elucidated here occur prior to birth and others after.

Vision continues to refine postnatally in most mammals. Human infants acquire a similar acuity and depth of vision as adults only by six months of age. Unlike human newborns, rodents, lagomorphs (rabbits) and carnivorous mammals have their eyelids sealed together at birth. Eyelid closure, i.e. fusion and opening are significant developmental events that direct corneal maturation. In human foetuses, the eyelids emerge first during 8th GW, close during the 10th GW and open around 24-27th GW (Eghtedari et al., 2016; Zieske, 2004). In mouse, the eyelids start to develop from the perioptic mesenchyme at E14.5 and close together in front of the cornea at E16.5 (Li et al., 2015). Mice that lack the expression of *Adam17*, *Egfr*, *Egr1* (with a BALB/c background) or *Fgf10* genes have postnatal ocular abnormalities, because of absent or dysfunctional eyelid development (Dong et al., 2017; Hassemer et al., 2013; Oh et al., 2017; Tao et al., 2005). These types of temporal differences between species is a characteristic feature of the developing vertebrate cornea and reflects both developmental and ecological divergence.

The epithelial surfaces of each organ and tissue exhibit a unique composition of keratin (Krt) molecules, which typically come in pairs of acidic and basic counterparts, based on the amino acid

sequence of the keratin. These keratins are an important support of the shape and strength in epithelial cells; they are the intermediate filaments that make up the cytoskeleton. The expression of keratins display well the process of epithelial cell differentiation. For example, suprabasal cells in adult skin epithelium express keratin pair *Krt1/Krt10*, whereas basal, less differentiated cells exhibit *Krt5/14* expression. Interestingly, before the eyelid is fused, the corneal epithelial cells do not express any keratins (Zhang et al., 2005). *Krt14* expression starts in the inner eyelid epithelium at E14.5 (in mouse) and expands promptly to the conjunctival and corneal epithelia after eyelid fusion at E16.5. The corneal-specific *Krt12* appears as early as E15.5 or E16.5 in scattered locations in the upper layer of the two corneal epithelial layers at this point (Tanifuji-Terai et al., 2006; Zhang et al., 2005). However, widespread expression is observed only after birth. The basic *Krt3* pair of *Krt12* is not expressed in mouse. In human, keratin expression follows a similar timing. First *Krt14*⁺ cells appear at the same time with eyelid primordia at 8th GW and *Krt3/Krt12*⁺ cells at 12th GW (Davies et al., 2009; Eghtedari et al., 2016; Rodrigues et al., 1987). It is possible that eyelid fusion creates a microenvironment that permits cellular differentiation in the ocular surface, not only in the cornea, but also in the conjunctiva and eyelid. This suggests that the amniotic fluid has an inhibitory effect on the ocular surface maturation.

The second developmental milestone in corneal maturation is the opening of the eyelid. When it comes to timing of the opening, the studied species divide to two groups. The eyelid opens either before birth, often the exact timing is hard to decipher, or postnatally, typically at P14. Eyelid opening induces extensive cell proliferation in the basal cell layer that could fuel a subsequent stratification of the epithelium (Francesconi et al., 2000). In human, eyelid opening takes place before birth, as well as epithelial stratification (Eghtedari et al., 2016). In mice and rats, where eyelid opens postnatally, the stratification starts at P14, when the third and fourth epithelial layers first emerge (Chung et al., 1992; Zieske, 2004). At P21, four to five layers are apparent and thereafter all the epithelial layers develop (Chung et al., 1992; Zieske, 2004). The process of stratification leads to changes in cellular morphology in the generated layers, so that the basal cells acquire their cuboidal shape and upper layers become more flattened (Zieske, 2004). Lack of one *Pax6* allele (*Pax6*^{+/-}) results in the loss of corneal stratification (Davis et al., 2003). Furthermore, intricate signalling via *Bmp4* between the stroma and epithelium is necessary for stratification (Zhang et al., 2015). Notably, even adult corneal, epithelial cells do not generate a cornified layer under normal circumstances, such as skin cells do.

While the corneal epithelium matures, the stroma and endothelium undergo significant morphogenetic events as well. The corneal stroma first swells and then thins to finally achieve its adult thickness and clarity (Song et al., 2003). Furthermore, both stromal and endothelial cells finish cycling and stop in cell cycle phases G0 and G1, respectively (Zieske, 2004).

Drastic changes in corneal epithelial gene expression patterns follow eyelid opening. Norman and her co-workers compared the transcriptomes of pre-opening (P9) and post-opening (6 weeks) mouse corneas and found out that 34% (P9) and 27 % (6 weeks) of transcripts were unique to the time points (Norman et al., 2004). In addition, both human and mouse display a gradual shift of some gene expression domains after eyelid opening. $\alpha 9\beta 1$ integrin, *p63* and *Krt15* are all expressed in the entire epithelium before, but afterwards only in the peripheral cornea (Davies et al., 2009; Pajoohesh-Ganji et al., 2004). All in all, these changes indicate that eyelid opening imposes a dynamic shift in corneal gene expression.

The keratin markers of mouse cornea display a particularly interesting maturation pattern. *Krt14* expression is spread over the entire corneal epithelium at birth, but then slowly disappears from the central cornea through desquamation and remains only in basal cells of the peripheral cornea (Di Girolamo et al., 2015; Richardson et al., 2017). Interestingly, this does not coincide with eyelid opening, but occurs much later, around 5 weeks of age (Pajooohesh-Ganji et al., 2016; Richardson et al., 2017). Also, mouse *Krt12* expression, a hallmark of terminal differentiation of the corneal epithelium, attains the adult expression domain quite late. Embryonic and early postnatal *Krt12* expression is localized to the upper cell layer, however, by P30, the positive cells encompass all corneal epithelial layers (Tanifuji-Terai et al., 2006). Remarkably, the entire cornea is *Krt12*⁺ at 6 months of age, suggesting that in mouse, the process of corneal maturation continues into adulthood (Tanifuji-Terai et al., 2006). Terminal differentiation and maintenance of corneal fate is inhibited in the absence of *Pax6* expression (Li et al., 2015; Ouyang et al., 2014). Ultimately, maturation shapes, shifts and arranges the corneal cells so that the peripheral and central cells separate to different populations and perform distinct tasks in corneal homeostasis.

2.2.4. Constructing the stem cell niche

The spatial separation of the peripheral and central corneal cells also manifests in the morphology of these two regions. The peripheral cornea turns into a housing for the corneal epithelial stem cells and is referred to as the limbus, i.e. “border” in Latin. Indeed, limbus develops in the junctional zone between the cornea and conjunctiva and makes up a barrier between these two tissues. The stepwise development of the limbus has been investigated in human foetuses. First, the limbal dome, a thickening of the limbal epithelium outwards, appears at 12th GW and then turns into a prominent ridge-like structure by 17th GW (Davies et al., 2009; Eghtedari et al., 2016). By birth, the ridge is lost and replaced postnatally by the palisades of Vogt, epithelial invaginations into the limbal stroma that characterize the limbus. The palisades offer sheltered “pockets” or crypts for the LSCs (Dua et al., 2005). In addition to human, the LSCs localize to the basal epithelium of the palisades of Vogt in rabbit and pig. However, mouse and rat limbus do not feature the palisades and as a result there are no clear boundaries to the limbus. Instead, the murine limbus is defined by its gene expression network. Some of the genes discussed in the previous section (section 2.2.3.) show limbal localization after maturation and mark the undifferentiated cells. However, all limbally expressed genes do not mark LSCs, instead some of them might contribute to the maintenance of the limbus as a stem cell niche.

The limbus has several anatomical features that the central cornea lacks. The limbal stroma is vascularized; arterioles encompass the palisades and thus expose the stem cells to systemic factors (Van Buskirk, 1989). In addition, the limbus contains the main outflow channels of the aqueous humour fluid to the blood circulation (Van Buskirk, 1989). There are also immunoresponsive Langerhans cells in limbal stroma and epithelium (Vantrappen et al., 1985). The basal limbal epithelium houses melanocytes, which support self-renewal of the LSCs, but might also provide a layer of protection from UV radiation (Dziasko et al., 2015). The limbus is a complex tissue that performs a multitude of functions that would not be possible in the central cornea without compromising its transparency.

2.2.5. Homeostatic renewal of the cornea

The cornea renews continuously throughout life. Early work identified the LSCs by label retention and defined the limbus as the corneal stem cell niche (Cotsarelis et al., 1990; Lehrer et al., 1998). Colony formation assay confirmed that the limbus contains the least differentiated cells of the cornea, because limbal samples gave rise to holoclones *in vitro* (Pellegrini et al., 1999). Since these early findings, researchers have struggled to localize the expression of stem cell marker genes in the LSCs. Many of the genes that denote corneal maturation, such as *Krt14*, *$\alpha 9\beta 1$* integrin and *p63* would be useful, but they are likely expressed in both stem and progenitor cells. Several putative markers have been proposed and few of them are discussed here. The murine, limbal *Krt15*⁺ cells have considerable stem cell properties in renewal and wound healing (Nasser et al., 2018). The expression of C/EBP δ marks the LSCs in human and interestingly, it is expressed together with the well-known stem cell marker gene *Bmi1* (Barbaro et al., 2007). Human limbal cells express also ABCG2 (Chen et al., 2004; Dua et al., 2005; Schlötzer-Schrehardt and Kruse, 2005). These cells exhibit a higher colony formation efficiency compared to *Abcg2*⁻ cells, suggesting that this might be a putative stem cell marker in human limbus (de Paiva et al., 2005). Another promising marker gene is ABCB5, which was identified in human and mouse limbus (Ksander et al., 2014). Rigorous experiments showed that the *Abcb5*⁺ cells are LRCs, they restore new cornea upon transplantation and knockdown of *Abcb5* expression causes defects in corneal differentiation and wound healing (Ksander et al., 2014). These indications suggest that *Abcb5* could be used to mark the LSCs. Lineage tracing studies to elucidate cellular dynamics and hierarchy would give further confirmation. Perhaps self-evidently, the limbal location of the stem cells is supported by the lack of differentiation markers *Krt3/12* (Schermer et al., 1986). However, more recent evidence suggests some overlap between the limbal stem cells and the *Krt12*⁺ corneal cell population (Kasetti et al., 2016). An option is to utilize co-expression of a few putative stem cell markers in combination with the lack of differentiation markers as a signature for the LSCs.

The renewal of the corneal epithelium is fundamentally different from other renewing epithelia. Because the limbus resides in the periphery of the cornea, new epithelium is produced centripetally, i.e. from the periphery to the centre. In contrast to, for example epidermis and intestine, where stem cells reside below the differentiated part of the tissue and produce new cells vertically from the bottom towards the surface. Corneal renewal follows the XYZ hypothesis that was formulated by Thoft and Friend based on renewal dynamics of the cornea (Thoft and Friend, 1983). First, LSCs divide in the basal epithelium (X). Then their immediate daughter cells, the corneal progenitors, migrate to the central cornea (Y) and finally dead cells desquamate from the surface (Z). Renewal is balanced so that $X+Y=Z$. The LSCs proliferate approximately every two to three days (Beebe and Masters, 1996). Progenitor cells divide further once or twice in the basal and suprabasal layers (Lehrer et al., 1998). Interestingly, the progenitors leave the basal layer as pairs of two cells that undergo terminal differentiation together while they encompass all the epithelial layers (Beebe and Masters, 1996). This process takes more than two weeks (Haddad, 2000). At the same time, the differentiated cells move horizontally towards the central cornea. This was elucidated with *LacZ*⁺ and *LacZ*⁻ chimeric mice (Collinson et al., 2002). X-Gal staining of the *LacZ*⁺ tissue reveal radial stripes that extend from the limbus to the centre (Collinson et al., 2002). The stripes maintain throughout life, so they must be continuously generated by the LSCs.

The activity of the LSCs was subjected to lineage tracing with a ubiquitously expressed CAGG-driver and, more restrictively, with *Krt14* driven expression of Confetti (Brainbow 2.1) reporter (Amitai-Lange et al., 2015; Di Girolamo et al., 2015; Dorà et al., 2015). *Krt14* is a good choice for lineage tracing, because of its basal, limbal expression site in adult mice and the possibility to induce reporter activity at any given time (Di Girolamo et al., 2015). The latter property allows for analysis of the temporal aspect of corneal stripe formation. These studies confirmed the work of Collinson and his colleagues, giving another set of evidence for the localization of the LSCs and the centripetal renewal pattern. However, these studies did not investigate why and how the differentiating, epithelial cells are forced away from the limbus. A mathematical simulation model provided some explanation to the cause and speed of centripetal renewal (Lobo et al., 2016). The model implies that the stem cells, located in the corneal periphery, are unable to move from that location creating a higher population pressure in the limbus. Whereas progenitor cells are migratory and exit the high-pressure region and enter lower pressure region in the central cornea. This explains the centripetal pattern without any external cues (Lobo et al., 2016). Centripetal renewal from the periphery to the centre takes up to 5 months, but it speeds up upon injury (Amitai-Lange et al., 2015; Di Girolamo et al., 2015). This shows that homeostatic renewal of the corneal epithelium is a slow process, but it has flexibility upon corneal trauma.

Interestingly, the chimera and lineage tracing studies on mouse detected an age-dependent switch in corneal renewal patterns. Before the radial stripes emerge, the labelled cells form a dotted pattern suggesting that the epithelium is renewed locally by basal cells of the central cornea (Collinson et al., 2002; Dorà et al., 2015; Mort et al., 2009; Richardson et al., 2017). This resembles epidermal renewal by the proliferative unit, where a basally located stem cell generates the epidermal cells just above itself (Potten, 1974a). Knowledge on corneal maturation and *Krt14* expression suggests that at first the corneal stem cells localize in the central cornea and only later, during maturation, home into the limbal niche. This is demarcated by the shift of *Krt14* expression from the centre to the limbus (Pajooresh-Ganji et al., 2016; Richardson et al., 2017). Similar switch from morphogenetic to homeostatic renewal was recently documented in the sebaceous gland and the intestine (Andersen et al., 2019; Guiu et al., 2019). Another explanation is that the central, corneal progenitor cells have considerable proliferation potential and can maintain the epithelium for a limited time. Serial transplantations of various parts of the cornea indicate that the central corneal tissue restores the entire epithelium in the case of injury and renews the epithelium just as well as the limbal tissue (Majo et al., 2008). The mice used in these experiments were 4 months old and thus, the outcome cannot be attributed to an age-dependent phenomenon. At the moment the importance of this work is not clear. Regardless, the age-dependency in corneal renewal is an essential feature to keep in mind when planning studies on corneal stem cells. Central, vertical renewal pattern moves over and is replaced by the limbal, centripetal renewal sometime around 7-8 weeks of age (Collinson et al., 2002; Mort et al., 2009; Richardson et al., 2017). However, it will take several weeks before the corneal stripes are fully grown. Of note, all these experiments were completed with mouse, leaving a possibility that this is a species-specific phenomenon. Indeed, the colony formation potential of the limbal and central corneal epithelial cells varies between species (Majo et al., 2008). This reveals the need for more experiments on corneal renewal at different ages and with different model organisms.

What about the mode of stem cell divisions in the cornea? Does it follow the asymmetrical or symmetrical pattern? As a matter of fact, there is no unified agreement on cornea. However, there are

pieces of evidence that point towards the symmetrical model of renewal. Interestingly, the Confetti reporters used to study centripetal renewal showed that each stripe consisted of cells labelled with the same colour; meaning that the cells in each stripe originate from the same stem cell clone (Amitai-Lange et al., 2015; Di Girolamo et al., 2015). This does not directly imply symmetrical divisions, but over time the stripes become fewer and wider, which then is a hallmark feature of symmetrical division pattern (Collinson et al., 2002; Dorà et al., 2015; Klein and Simons, 2011; Mort et al., 2009).

The limbal stem cell niche is a vital part of corneal homeostasis. The stroma and the BM of the limbus present the overall microenvironment to the epithelial niche (Chen et al., 2019; Notara et al., 2010; Nowell and Radtke, 2017). The stroma houses keratocytes and a small population of fibroblasts that secrete growth factors to the limbal epithelial cells. The BM in limbus contains various isotypes of laminins, collagens as well as vitronectin; proteins that are not present in the central region of the BM (Dziasko and Daniels, 2016). Thus, the stroma maintains an appropriate molecular, mechanical and cellular environment for the limbal niche.

Limbal epithelium itself participates as well in stem cell maintenance. The expression of *Pax6* by the limbal epithelial cells is necessary to maintain the corneal fate (Li et al., 2015). In the case of *Pax6* knockdown, the limbal cells transdifferentiate to resemble epidermis via loss of *Wnt7a* signalling (Li et al., 2015; Ouyang et al., 2014). In addition, *Pax6* expression maintains an intricate downstream signalling network, where members of the TGF- β family play a prominent role in corneal, epithelial cell fate maintenance (Li et al., 2015).

Aberrant niche microenvironment or altered gene expression profile can lead to drastic problems in corneal homeostasis. Limbal stem cell deficiency (LSCD) is a pathology where the stem cells are partially or fully lost due to injury, disease or a congenital defect. In many patients, LSCD results in neovascularization and conjunctivalization; vasculature and cells of the conjunctiva invade the cornea, respectively. In these cases, the maintenance of the limbal niche is compromised and it no longer acts as a barrier between the cornea and the conjunctiva. Severe conjunctivalization leads to loss of vision because the cornea becomes opaque. Interestingly, experimental ablation of the limbal epithelium does not cause LSCD in mice, but initiated renewal from the central cornea towards the limbus (Nasser et al., 2018). This suggests a mechanism for acute replacement of the LSCs and re-establishment of the limbal niche maintenance.

The corneal stroma is composed of differentiated keratocytes and fibroblasts. In addition, a small population of stromal stem cells that resemble MSCs resides in the limbal region of the stroma (Du et al., 2005; Polisetty et al., 2008). The stromal stem cells encompass a multitude of stem cell characteristics, including self-renewal and high colony-formation efficiency. Furthermore, they are multipotent as stromal stem cells can be directed to keratocyte, chondrocyte or neural fates with proper inductive media (Du et al., 2005). In human, the stromal stem cells localize to the ripples and folds of the palisades of Vogt, in direct contact with the LSCs (Du et al., 2005; Dziasko et al., 2014). They share some of the putative markers with the LSCs, like *Abcg2* and *Bmi1*, but express indifferent markers like *Ssea4*, *Six2*, *Fhl1*, *Thy1* and *Nestin* as well (Du et al., 2005; Funderburgh et al., 2016; Kaplan et al., 2019). Most researchers presume that the stromal stem cells do not replace the quiescent, stromal keratocytes under homeostasis. Instead, they are limbal support cells and an essential part of the niche microenvironment to the LSCs (Chen et al., 2011).

In contrast to the epithelium and the stroma, the deepest layer of the cornea, the endothelium, does not renew itself, not even in the case of an injury. Differentiated endothelial cells exit the cell cycle and do not regenerate the cell layer. Yet, the endothelium contains a population of cells, that

have stem cell -like characteristics, suggesting that some cells do maintain a higher proliferative potential than suspected (Amano et al., 2006; Yam et al., 2019).

2.3. Lacrimal gland

The ocular surface requires constant lubrication to maintain a viable epithelium. A transparent, acellular tear film provides a hydrating and smooth surface over which the eyelids can glide effortlessly to remove microbes and small particles that otherwise might be harmful to the eyes. The tear film is only 3-7 μm thick but consists of three layers. The deepest layer, closest to the ocular surface, is called the mucous layer and it is mainly produced by the goblet cells of the conjunctiva, but the corneal epithelial cells also secrete mucin proteins that contribute to the film (Gipson and Argüeso, 2003; Woodward and Argüeso, 2014). A large, aqueous layer coats the mucous part. LGs, located in the region between the eye and the ear, secrete water, electrolytes, growth factors, and even some mucins to this layer (Klenkler et al., 2007). This is of particular importance because the corneal epithelial cells reside too far to obtain nutrients and oxygen from the aqueous humor. Thus, the aqueous part of the tear film is a likely source of these essential ingredients to the epithelial cells. Lastly, the outermost, lipid layer gives a thin coverage for the entire tear film. The lipids are produced in the meibomian glands, which reside in the eyelids. In addition to lubrication and physical barrier, the tear film presents anti-microbial protection to the ocular surface (McDermott, 2013). Together, the movements of eyelids and the tear film provide another level of protection to the ocular surface.

The LGs are present bilaterally; there is a LG supporting each eye. The organ itself is divided into two parts; the intraorbital and extraorbital lobe, based on their location. Intraorbital lobe is located close to the eye, whereas the extraorbital lobe is situated further away, in proximity to the ear. There are species-specific differences in the size, amount and exact location of the LGs (Obata, 2006; Schechter et al., 2010). However, in this thesis I focus on the murine extraorbital LGs. The LG has a typical morphology of an exocrine gland; a secretory tissue is shaped like a tree that spreads into connective tissue, where the tree branches, together with nerves and vasculature, protrude into ECM. Ducts, acini and myoepithelial cells make up the epithelial compartment, i.e. the “tree”, of the LG (Fig. 6).

The ducts comprise two epithelial cell layers, the basal and the luminal, the latter facing the lumen of the duct. The epithelial cells of the LG ducts modify the lacrimal fluid before it is secreted (Katona et al., 2014). The principal duct connects the LG to the ocular surface, whereas smaller ducts house acini, end units of the ducts that are the secretory regions of the LG. Acinar cells have a distinct, pyramid-like shape and produce the tear fluid in secretory vesicles that empty to the lumen of the ducts. In comparison to other glands, the LG has an abundance of acini versus ducts; 80 % of the LG volume is made of acinar cells (Schechter et al., 2010). Myoepithelial cells are part of the epithelium, but resemble smooth muscle cells and thus help the release of secretory products from the acinar cells with contractile cell processes (Makarenkova and Dartt, 2015).

The secretion of tear fluid by the LG is referred to as lacrimation or tear production. Regular lacrimation is characterized by basal tears, which are continuously produced and present on the ocular surface. However, the LGs secrete also reflex tears in case of a chemical or physical stimulus or harm on the ocular surface (Murube, 2009). In addition, psycho-emotional lacrimation occurs after emotional stimulus (Murube, 2009). Movements of eyelids sweep away excessive tears and collect them in the lacrimal sac that connects to the nasal cavity.

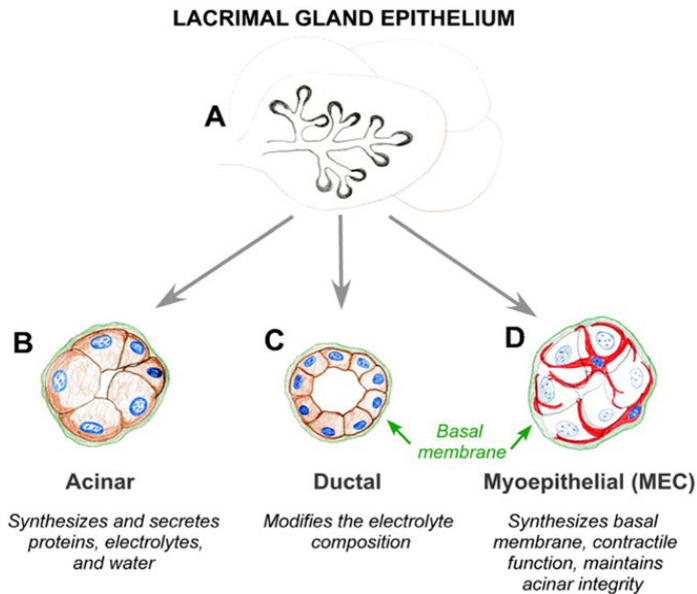


Figure 6: The epithelial compartment of the LG.

(A) The epithelium of the LG forms a tree-like organ structure that sits in connective tissue. (B) The branch tips have acinar, secretory cells. (C) Ductal cells compose the ducts and (D) myoepithelial cells (in red) enfold the acinar cells with long cell processes. Image from Makarenkova and Dartt, 2015. Reprinted with permission from Springer Nature.

2.3.1. Development of the LG

The LG develops from the ectoderm, similarly to the other parts of the eye. The development of the eye occurs before LG morphogenesis and the latter initiates only when the general ocular structures are in place. Extraorbital LG lobe formation starts from the surface ectoderm that represents the developing conjunctival epithelium and is located on the temporal side of the eyeball (Kuony and Michon, 2017; Makarenkova et al., 2000). First, at E12.5 in mouse, the epithelium thickens and forms a bud-like shape (Fig. 7)(Makarenkova et al., 2000). Next, the bud starts to elongate, forming a principal duct that extends towards the developing auditory region. The formation of the LG ductal tree follows a morphogenetic process called branching morphogenesis. Here, a simple epithelial tube remodels to a network of tubules through several subsequent divisions of the tube. Branching of the LG epithelium begins at E16 and continues postnatally (Kuony and Michon, 2017; Makarenkova and Dartt, 2015; Makarenkova et al., 2000).

Interestingly, branching morphogenesis of the LG seems to be fuelled by rearrangement of the ductal epithelial cells and proliferation occurs mostly in the branch tips (Kuony and Michon, 2017). During branching, the LG ducts are filled with epithelial cells that are removed by apoptosis (Kuony and Michon, 2017). Thus, apoptosis makes space for the ductal lumen. Each branch finishes in a terminal end bud where the acinar compartment develops. The intraorbital lobe of the murine LG forms as a side-branch from the elongated LG principal duct at E17.5 and follows branching morphogenesis after that (Makarenkova et al., 2000). Thus, the intraorbital lobe is located closer to the eye due to later initiation of development. Interactions between the epithelium and underlying mesenchyme define LG development. A mesenchymal capsule surrounds the forming LG throughout development and provides a necessary signalling environment for LG development. FGF7 and FGF10 from the mesenchyme signal to the epithelium and initiate LG formation (Makarenkova et al.,

2000). Furthermore, FGF10 and BMP7 regulate the mesenchymal environment by rearrangement of the ECM, induction of proliferation and condensation (Dean et al., 2004; Tsau et al., 2011). FGFs expressed in the mesenchymal capsule guide branching morphogenesis and define ductal length and terminal end bud location (Thotakura et al., 2019). Later, the mesenchyme remodels, forming the connective tissue that surrounds the adult LG.

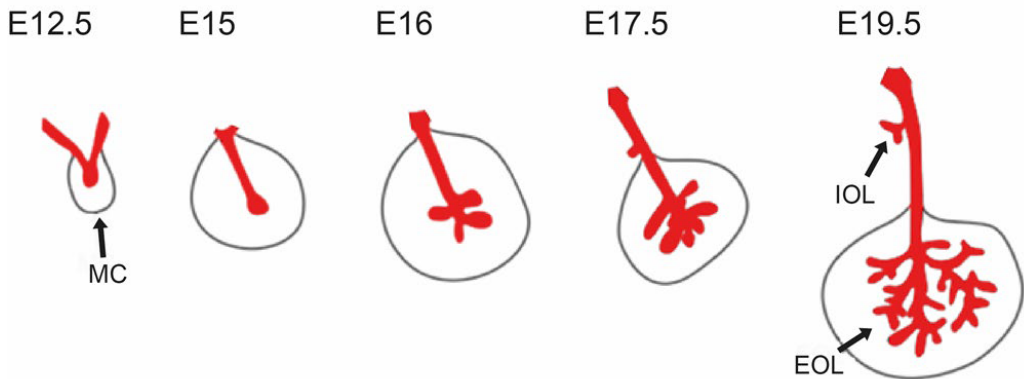


Figure 7: Development of the LG. LG epithelium (in red) derives from a hollow in the developing conjunctival epithelium at E12.5, when it forms a bud. The bud extends to a mesenchymal capsule (MC) and starts to branch at E16. At E17.5 the bud of the intraorbital lobe (IOL) appears from the principal duct of the extraorbital lobe (EOL). Branching continues postnatally.

2.3.2. Maturation and maintenance of the LG

Postnatal maturation defines the function of the LG as a secretory organ. As in cornea, the timing of maturation follows the timeline of eyelid opening. Thus, the species-specific differences reflect maturational processes here as well. In human, LG maturation likely occurs before birth, however in mice it takes place postnatally (Farmer et al., 2017). Before eyelid opening, the acinar cells of the LG do not produce tear fluid and thus, the cornea does not have a protective tear film. However, after eyelid opening the film forms quickly, as evidenced by the activation of mucin-producing goblet cells (Watanabe et al., 1993). A large-scale gene expression study on embryonic and postnatal LG suggests that the expression of a multitude of genes changes upon maturation (Farmer et al., 2017). Developmentally important genes are downregulated soon after birth and replaced by genes that display maturational processes and even later on, adult secretory functions (Farmer et al., 2017). Interestingly, some genes, like keratins, are highly expressed at the time of birth (Farmer et al., 2017). In addition to modification of the gene expression network, gross morphological events take place as well. Branching morphogenesis completes by P30 (Makarenkova and Dartt, 2015). In mouse, both LG lobes grow extensively and reach their adult size as late as by P50 indicating that LG maturation is a long process (Kuony and Michon, 2017).

How is the LG maintained in adulthood? Interestingly, definitive stem cells of the LG are not characterized, however the gland has a remarkable regenerative potential suggesting that it indeed contains stem cells (Zoukhri et al., 2007; Zoukhri et al., 2008). Label retainment revealed that each compartment of LG; the acini, ducts, myoepithelium and the even the connective tissue contain LRCs

(You et al., 2011a; You et al., 2011b). Furthermore, these LRCs become proliferative upon LG injury. Lineage tracing of the *Krt5*⁺ ductal cells showed that basal cells of the duct generate both basal and luminal progeny and thus serve as the ductal stem cell population (Farmer et al., 2017). To date, other stem cell populations of the LG have not been studied with lineage tracing. However, Gromova together with her colleagues suggested that a common, epithelial progenitor exists in the LG and that might contribute to the healing potential of the LG (Gromova et al., 2017). Molecular analysis of adult LG cell populations is necessary to identify stem cell marker genes in the different cell populations within the tissue.

2.3.3. The connections between cornea and the lacrimal gland

The LG is a vital part of corneal maintenance and vice versa. These two tissues communicate with each other utilizing two, very different mechanisms. The LG regulates the corneal epithelium via the tear fluid, whereas cornea signals to the LG through neural stimulus. The tears provide an abundance of factors to the cornea. Among the necessities, nutrients, oxygen, and mucin, a multitude of growth factors and signalling molecules are secreted into the tear fluid and enter cornea. One of these factors is EGF, which has a proliferative effect on corneal epithelial cells (Imanishi et al., 2000). Other, known factors include TGF- β family members, HGF, PDGF, VEGF and neurotrophins (Klenkler et al., 2007; Yoshino et al., 1996a). Most of these have the potential to modulate corneal cell proliferation and/or movement (Grant et al., 1992; Imanishi et al., 2000; Song et al., 2002). The contents of the tears changes depending on the type of lacrimation, basal or reflex (van Setten, 1990). After corneal insult, the LG specifically secretes clusterin-protein to the tear fluid that subsequently accelerates corneal healing response (Fini et al., 2016). Furthermore, inflammatory responses spread through the corneal surface via tear film, perhaps providing a rapid mechanism to respond to an insult (Nishida et al., 2015). Analysis of tear contents is a useful tool to diagnose the ocular surface or LG diseases as well.

Lacrimation is a one-way route from the LG to the cornea. Information from the cornea to the LG is transmitted through a nervous pathway. Cornea is highly innervated and as a result, very sensitive. Large nerve bundles infiltrate the corneal stroma from the periphery to the centre. Smaller nerve endings penetrate the Bowman's membrane and extend to the epithelium, where they gather in a vortex-like pattern while intermingling with the epithelial cells (McKenna and Lwigale, 2011). Any sensory stimulus, whether chemical, mechanical or thermal, transduces immediately through the afferent, corneal nerves. These nerves join the ophthalmic branch of the trigeminal nerve just outside the eyeball. The trigeminal nerve gathers the corneal nerves together with the efferent neurons that lead to the LG. Nerves from the parasympathetic system innervate the secretory acini of the LG. Ablation of the parasympathetic nerves reduces tear production due to lack of basal, inductive signals from the nervous system (Toshida and Suto, 2018). Corneal insult causes reflex tear production from the LG immediately.

2.4. Corneal defects

Being a superficial tissue, the cornea is easily challenged by insults from the external environment. Sources of injuries are diverse, often caused by small particles such as dust or sand, scratches, or other foreign objects that abrade a part of the corneal epithelium. In fact, corneal abrasions are the most common eye insult in the emergency care (Jackson, 1960). Other sources of corneal injuries

include chemical and thermal burns, as well as less-frequent UV or other radiation exposure. In addition to being subject to a potential loss of vision, large corneal trauma is painful to the patient.

External insults on the cornea can lead to an ocular surface disease. These diseases encompass a multitude of conditions, but dry eye disease (DED), which is caused by either decreased tear production by the LG and/or increased fluid evaporation, affects 7-33 % of the population (Gayton, 2009; O'Brien and Collum, 2004; WHO, 2019). As a result, the tear film is unstable and unable to maintain a protective layer on top of the ocular surface. Patients experience a dry sensation, together with a general feeling of discomfort, burning, stinging or grittiness in the eye. Even though DED can be temporary and mainly caused by environmental conditions, the risk of developing a chronic DED increases with age (Farrand et al., 2017). Furthermore, laser-associated corneal refractive surgery leads to DED in almost all patients (Shtein, 2011). A combination of DED and exposure to UV radiation and dust can generate the formation of pterygium, which is a visible outgrowth of the conjunctiva towards the cornea. Other ocular surface diseases include allergic conjunctivitis; an inflammation of the conjunctiva that displays as redness of the eye and an irritating sensation. Contact lens wear can induce a bacterial infection of the cornea, called keratitis (Liesegang, 1997). All the above-mentioned diseases are treatable, but without proper help can lead to more severe conditions and loss of vision. In addition to these diseases, there are several congenital pathologies of the cornea. Treating these might require larger operations, such as corneal transplantation or prosthesis and reduce vision long-term.

2.4.1. Artificially induced corneal injury

Due to a high prevalence of ocular surface diseases, there is a need for adequate understanding of corneal injuries and healing. Thus, the cornea is a useful target for wounding and wound healing studies. There are numerous studies on cornea that use different types of corneal injuries. This reflects the variability of corneal insults in patients. The type of injury largely affects the mode, speed and extent of corneal healing (Stepp et al., 2014). Thus, when studying corneal healing, the choice of experimental injury type is instrumental.

A common type of artificially induced corneal injury is by debridement, where corneal epithelium is abraded, but deeper layers of the cornea remain intact. Later on, in this thesis, I describe the abrasion method in detail (section 5.4.). Abrasion is an excellent choice to mimic the most common corneal insults. Another injury model is the incisional wound. Here researchers use a surgical knife to generate a stromal or full-thickness wound through the entire cornea (Blanco-Mezquita et al., 2013; Kato et al., 2003). This model is used to study healing from eye surgery, where an incision through the limbus is used to reach lens and retina for the actual operation. Keratectomy wound is a partial thickness injury where the trauma is targeted to the stroma with a laser or manually using a surgical blade (Azar et al., 1998; Kato et al., 2003). Here, the aim is to induce minimal trauma to the epithelium and thus reveal the healing process ongoing in the stromal layer. Large and penetrating injuries challenge the stability of the entire cornea and might result in the collapse of the tissue. A well-established model of corneal injury is the alkaline burn with sodium hydroxide (NaOH) with or without filter paper on the corneal surface (Bai et al., 2016). Of note, using a filter paper to induce a chemical injury induces a mild mechanical injury, impression, in addition to the chemical treatment. Chemical exposure with an alkaline results in a large and diffuse injury that affects not only the corneal epithelium, but also the conjunctiva and stroma (Bai et al., 2016; Chan et al., 2013).

Strong alkaline solutions have been shown to induce corneal ulcers, opacification, and neovascularization (Bai et al., 2016). Thus, alkaline injury is an advisable method in studies related to stromal activation. Another type of chemical injury can be inflicted by applying dimethyl sulfoxide (DMSO) on the cornea (Amitai-Lange et al., 2015).

2.4.2. Ectodysplasin-A in corneal health

Ectodysplasin-A (Eda) is a developmentally important gene that initiates the formation of ectodermal appendages, such as hair, mammary gland, and tooth (Häärä et al., 2012; Shirokova et al., 2013; Voutilainen et al., 2015). The *Eda* gene belongs to the tumor necrosis factor superfamily of immune response proteins (Mikkola et al., 1999). However, the known EDA functions relate only to ectodermal differentiation. EDA enhances placode (first stage of appendage development) formation by increasing cell motility and the amount of placode-committed cells (Mustonen et al., 2003). This gene is located in the X chromosome and produces at least two functional isoforms, one of which (EDA-A1, here referred to as EDA) is secreted out of the cell and binds to EDAR receptor in a target cell (Elomaa et al., 2001). EDA-EDAR binding starts a signalling cascade, where a cytosolic adaptor molecule, EDARADD, cleaves from the plasma membrane and induces another cytosolic/nuclear factor, NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), to activate target genes of *Eda* signalling (Mikkola, 2009). In human, loss of any crucial member of the *Eda* signalling pathway causes hypohidrotic ectodermal dysplasia (HED), where the patients have missing or hypoplastic ectodermal appendages (Bayés et al., 1998; Döffinger et al., 2001). The clinical features of the condition include absent or missing teeth, hair, inability to sweat and dryness of skin and lungs, among many other issues. In fact, HED can lead to death, if it is not noticed early or diagnosed properly (Mikkola and Thesleff, 2003). A loss-of-function mutation in *Eda*, *Edar* or *Edaradd* in mice leads to a similar disease as in human (Headon and Overbeek, 1999; Headon et al., 2001; Kere et al., 1996; Srivastava et al., 1997).

As *Eda* is a critical gene in ectodermal appendage development, it is presumable that it has a role in cornea and LG development as well. Recently, *Eda* was linked to corneal maintenance in adult mice (Li et al., 2017a; Li et al., 2018a). Interestingly, the knockdown of *Eda* results in DED due to absence of meibomian glands (Wang et al., 2016). Subsequently, there is no lipid layer in the tear film and tear evaporation is increased. Furthermore, corneal pannus (i.e. neovascularization and opacification) is another ocular symptom of ectodermal dysplasia (Kaercher, 2004). The role of *Eda* in the development of cornea or LG has not been studied.

3. Aims of the study

Maintenance of a healthy cornea is essential for proper vision, because it acts as a barrier to pathogens and water-loss to the external environment. Corneal homeostasis is maintained by both cellular and extracellular components of the ocular surface; the corneal cells and the tear film. The main players here are the epithelial cells of the cornea and the LG secretion. Understanding the development, maturation and functional dynamics of these compartments contributes to the knowledge on the corneal barrier function.

The cornea provides an excellent tool to study wound healing. Corneal epithelium is regularly challenged by small particle injuries and scratches. The mode of healing after corneal injury is a largely disputed topic, partly due to the type of injury, its penetrance, and extent on the cornea. LESC's participate in wound healing only on specific types of injury, however the role of the central corneal epithelial cells in injury remain obscure. The molecular signals and growth factors involved in corneal wound healing response have been studied in detail (Klenkler et al., 2007). A likely source of these signals is the LG. However, how corneal response to injury or DED transmits to LG functionality is not self-evident.

The aims of this thesis are to:

1. Observe the dynamics of the *Bmi1*⁺ cells in the murine corneal epithelium
2. Optimize a protocol for corneal abrasion using live mice
3. Elucidate the role of *Eda* during LG development and maturation
4. Understand the communication between cornea and LG in homeostasis and DED

4. Material and methods

Mouse strain	Reference	Used in study
<i>Bmi1^{CreERT}</i>	(Sangiorgi and Capecchi, 2008)	I
C57BL/6JRccHsd	Envigo	III
<i>Eda^{-/-}</i>	(Falconer, 1952; Srivastava et al., 1997)	III
Hsd:ICR(CD-1)	Envigo	I
NF-κB-gal	(Bhakar et al., 2002)	III
NMRI	Envigo	I, II
<i>R26R^{lacZ}</i>	(Soriano, 1999)	I

All aspects of mouse care and experimental protocols required for the completion of this thesis were approved by the Finnish National Board of Animal Experimentation.

Method	Reference	Used in study
Alkaline burn	II	II
Cell proliferation assay (BrdU)	I	I
Cell proliferation assay (Ki67)	I	I
Corneal abrasion	II	I, II, III
Eye and cornea measurements	I	I
Fluorescein staining	II	I, II, III
Genetic inducible lineage tracing	(Sangiorgi and Capecchi, 2008), I	I
Haematoxylin and eosin staining	(Juuri et al., 2012), II	II, III
Immunofluorescence	I, III	I, III
Immunohistochemistry	I	I
LG blinking rate analysis	III	III
LG tear volume analysis	III	III
LG weight analysis	III	III
Quantitative PCR	III	III
RNA <i>in situ</i> hybridisation (RNAscope)	(Wang et al., 2012), I	I, III
RNA sequencing	III	III
Tamoxifen administration (intraperitoneal and oral gavage)	I	I
X-Gal staining	I, III	I, III

Antibodies	Host species	Company and product	Used in study
β -CATENIN	Mouse	BD Biosciences, 610154	I
BMI1	Rabbit	Abcam, ab38295	I
BMI1	Rabbit	Cell Signaling, 6964S	I
BRDU	Mouse	GE Healthcare, RPN202	I
E-CADHERIN	Mouse	BD Biosciences, 610182	III
KERATIN 19	Rabbit	Abcam, ab52625	I
KERATIN 19	Rabbit	Abcam, ab5694	III
KI67	Rabbit	Abcam, ab16667	I
PHOSPHO-HISTONE H3	Rabbit	Abcam, ab5176	III

RNA <i>in situ</i> hybridisation (RNAscope) Probe	Probe number	Used in study
<i>Mm-Bmi1</i>	466021	I
<i>Mm-Eda</i>	423011	III
<i>Mm-Edar-C2</i>	506211-C2	III

5. Results and discussion

5.1. General eye growth and establishment of the adult gene expression pattern define corneal maturation (study I)

Studies on epithelial renewal and expression of keratin markers indicate that murine corneal maturation continues from birth far into adulthood. I addressed the role of corneal growth in maturation and determined that both the eyeball and the cornea grow postnatally, until 4 and 16 weeks of age, respectively. Eyeball and corneal growth did not correlate after 4 weeks, instead between 4-16 weeks, when the eyeball growth ceased, the diameter of the cornea still enlarged. In adult mice the cornea covers a larger proportion of the ocular surface than in human. In another study on ovine samples, the eyeball and cornea sizes correlated strongly (Doughty, 2017). Also in human infants the eye and cornea grow concurrently, however most of the rapid growth occurs during the first year of life (Augusteyn et al., 2012). In human, the eye reaches its final size (21-27 mm in diameter) in adolescence. The distance from the cornea to retina as well as corneal curvature affect the sharpness of vision, thus eyeball-to-cornea size might contribute to species-specific differences in vision.

Francesconi and co-workers suggested cellular proliferation as a key mechanism of postnatal maturation in rat corneas (Francesconi et al., 2000). I quantified the amount of proliferative, *Ki67*⁺ cells in newborn, juvenile and adult mouse corneas. My result did not verify the earlier observation on a significant proliferation peak at P14, instead I showed a stable maintenance of proliferation at all ages, supporting first maturation and later homeostasis. It is possible that the difference arises from different experimental animal model, however mechanisms other than proliferation likely contribute to corneal maturation as well. A multitude of local and systemic factors control organ growth and the mechanisms that signal on the completion of maturation, however their interplay in cornea remains to be elucidated (Conlon and Raff, 1999).

A change in keratin expression pattern illustrates the maturation of the cornea (Fig. 8). Previous studies described the opposite changes in expression domain for *Krt12* and *Krt14*. Differentiated cells of the cornea display *Krt12* expression and this domain encompasses the entire central cornea during maturation (Tanifuji-Terai et al., 2006). Whereas immature corneal cells are *Krt14*⁺, but expression is limited to the limbus postnatally (Di Girolamo et al., 2015; Richardson et al., 2017). I identified an additional keratin pair, *Krt8/Krt19*, in corneal maturation. I studied *Krt19* with immunofluorescence. However, for *Krt8* I chose the RNAscope method in order to visualize the presence of its mRNA in the cornea (Wang et al., 2012). RNAscope enables a more detailed visualization of the target mRNA and is thus a useful approach to study changes in the expression domain. At P0, immediately after birth, the entire corneal epithelium expressed both *Krt8/Krt19*. However, the basal epithelial layer seemed devoid of the *Krt8* mRNA. During the next maturational steps, both keratins became restricted to the upper of the two epithelial layers and were mostly lost from the central cornea at P14, when the eyelid opens, and epithelial stratification begins. The stratified epithelium (P21) did not express *Krt8/Krt19*. These results advocate for the use of *Krt8/Krt19* as markers of corneal maturation and the establishment of the limbal stem cell niche.

After maturation, *Krt19* expression remained prominent in the limbus, however not in the basal cell layer. In fact, some of the *Krt19*⁺ cells are also *Krt12*⁺, indicating that regardless of limbal location, they are terminally differentiated cells (Ramirez-Miranda et al., 2011). Supporting this observation, the *Krt8*⁺ cells in the murine limbus do not co-localize with the *Krt14*⁺ stem and/or

progenitor cells, suggesting that this might apply to the *Krt19*⁺ cells as well (Pajoohesh-Ganji et al., 2016). I propose that *Krt8/Krt19* expression in adult mice marks a subpopulation of cells that support the LSCs.

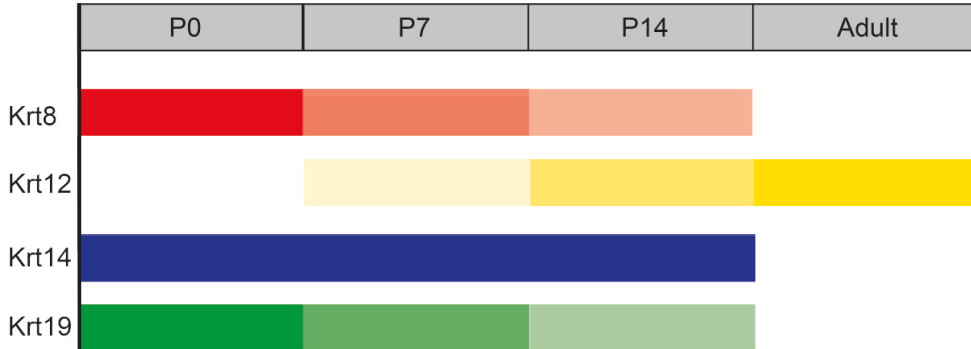


Figure 8: A summary of keratin expression changes in the murine central cornea during postnatal maturation. Strong colour indicates uniform expression and lighter colour signals for gradual change in expression domain. *Krt8*, *Krt14* and *Krt19* expression is lost upon maturation. At the same time *Krt12* expression becomes established in the central cornea.

5.2. *Bmi1* is expressed in corneal epithelial progenitor cells (study I)

The lack of consensus on the use of a stem cell marker gene in the murine cornea has led to a multitude of studies attempting to describe one. *Bmi1* associates with stem cell self-renewal and maintenance in many other tissues and in human, it is suggested as a marker of the LSCs (Barbaro et al., 2007). Given this knowledge on *Bmi1* in human cornea, I hypothesized that it might function in the murine LSCs as well. I determined that during development, BMI1 was present in all corneal layers, but after birth, only in the epithelium. In adult mice, *Bmi1* was expressed in central and limbal epithelium at both studied ages, 4 and 24 weeks. Because the expression domain encompasses both central and limbal regions, it is likely that in mouse cornea, *Bmi1* expression does not define the LSCs.

The result that *Bmi1* seems to not have a connection with the LSCs is in stark contrast with other studies on its function. Thus, I chose to analyze the dynamics of the *Bmi1*⁺ cells in the cornea. I employed an inducible genetic lineage tracing method with *Bmi1*^{CreERT/wt};*R26R*^{lacZ/wt} mice, resulting in *lacZ* expression after induction with tamoxifen. Here, cells that express *Bmi1* and their progeny are stained blue after a colorimetric reaction with X-Gal. Reporter expression was induced at 4 weeks of age and cornea samples were collected over a time course of 2, 4, 8, 12, 16, 20, 36 and 48 weeks after induction. At 2 weeks after, the *Bmi1* cell progeny displayed a patchy pattern of cell clusters. These cell clusters localized in central, peripheral, and limbal regions in equal frequencies (data was not quantified). Similar clusters have been shown with *Krt14* lineage tracing using a 10 day chase period (Amitai-Lange et al., 2015). My histological analysis confirmed that the clusters were multicellular and arose from *Bmi1*⁺ cells; the originally labelled cell located in the basal epithelium with vertically differentiating progeny just above it. Altogether, the labelled clone formed a cone-shaped lineage that resembles the EPU from skin (Potten, 1974b). With this set of experiments, I

indicated that epithelial turnover time from the basal layer to the surface takes between 2-8 weeks. This is in line with evidence from rabbit, where vertical turnover took more than two weeks (Haddad, 2000). In my work, cell clusters disappeared by 8 weeks after induction. Interestingly, vertical differentiation did not always occur in pairs, as suggested by Beebe and colleagues, but regarding this aspect our experiments remained inconclusive (Beebe and Master, 1996). In conclusion, these findings suggest that *Bmi1* is expressed in corneal, epithelial progenitor cells, because the *Bmi1*+ cells give rise to new epithelial cells *in vivo* and thus support corneal homeostasis.

Earlier studies have suggested that the proliferative, corneal epithelial progenitor cells localize mostly in the peripheral cornea, close to the limbus (Lehrer et al., 1998). This hypothesis has support from the centripetal renewal pattern that situates stem and progenitor cells in spatial hierarchy in limbus and corneal periphery, respectively (Collinson et al., 2002). However, I detected dividing cells in the central cornea by *Ki67* expression and BrdU labelling. In line with our observations, other genes that highlight the proliferative capacity of LSCs and progenitor cells, *Sox2*, *Sox9* and *p63*, do not have an exclusively peripheral expression domain (Bhattacharya et al., 2019; Menzel-Severing et al., 2018; Sartaj et al., 2017). However, others, like *Frizzled7* and *Importin13*, are expressed only in the peripheral or limbal regions of the cornea (Hayashida et al., 2010; Mei et al., 2014; Wang et al., 2009). Perhaps the corneal progenitor cells are a heterogeneous population of cells that hold a continuum of different proliferative capacities and this reflects their location and function in homeostasis.

Studies on *Krt14* lineage tracing showed first the appearance of corneal clusters in a patchy pattern, similarly as in our experiments. However, the clusters were replaced by limbal stripes in long term follow-up (Amitai-Lange et al., 2015). In my work, none of the collection points displayed the stripes. There were some rare, incomplete corneal stripes in our samples at 8, 12 and 16 weeks after induction. In support of that, in the histological sections I detected horizontal expansion of a few cell clusters. However, I presume that these stripes are rare, long-living progeny of the *Bmi1*+ progenitors that become subjected to the overall migration of the corneal epithelium that is forced by high population pressure in the limbus (Lobo et al., 2016). There were no full-length, continuously generated limbal stripes. This confirms that the *Bmi1*+ cell population does not encompass the LSCs. It is possible that the human limbal cells that express *Bmi1*, are rather a progenitor than a stem cell population. However, a functional analysis would give insight into this matter.

I do not fully understand the discrepancy between the *Bmi1* gene expression and lineage tracing outcomes, but one possibility is that the level of induction was lower than expected, thus not all *Bmi1*+ cells became labelled. Indeed, when using the inducible Cre-loxP method, some cells escape label due to dose-dependency, tissue-specific recombination efficiency or stochastic gene silencing (Dobie et al., 1997; Hayashi and McMahon, 2002). Of course, *Bmi1* expression might encompass both progenitor and differentiated cells. If some of the *Bmi1*+ cells did not undergo any cell divisions after labelling, they would hardly be detectable in the tissue. Also, some of the corneal progenitor cells have capacity to only one division (Lehrer et al., 1998). It is possible that a subpopulation of the *Bmi1*+ progenitor cells belongs to that category.

I, and others, have now provided evidence that the central cornea is maintained without limbal input, for a limited amount of time (Collinson et al., 2002; Dorà et al., 2015; Haddad, 2000; Mort et al., 2009). However, the age-dependency of the renewal pattern remains unclear. For this work, I used young, 4 weeks old mouse corneas, where maturation is complete, but the limbal renewal pattern not yet established. This might contribute to our results and analysis. A comparison of renewal pattern

between mice at 4, 12 and 24 weeks of age showed that younger animals displayed a higher frequency of central corneal clusters (Dorà et al., 2015). Richardson and co-workers dissected the age-related switch in detail and showed that when the (*Krt14*) lineage tracing begun at P7, there were full-length limbal stripes at the age of 6 weeks (Richardson et al., 2017). This is of particular interest, because it suggests that limbal renewal is ongoing already right after birth.

Corneal homeostasis is maintained by two, overlapping and equally important modes of renewal (Fig. 9). The vertical, temporally limited renewal by corneal epithelial progenitor cells and horizontal, slow renewal by the LESC. The progenitors have a relatively long-lasting proliferative capacity, but once this capacity is depleted, the progenitors become differentiated and eventually desquamate from the surface. LESC continuously generate new cells into the progenitor pool. Collectively, this data suggests that there is a higher quantity or activity of progenitor cells in young animals.

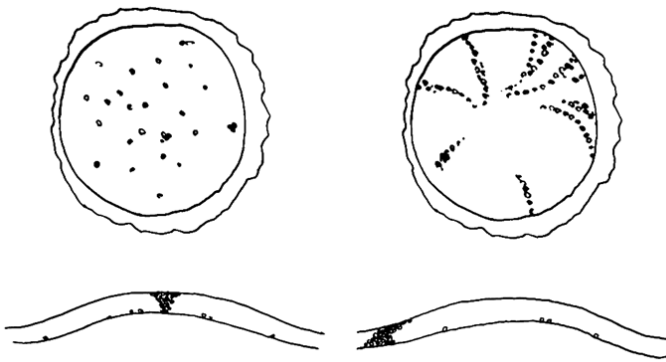


Figure 9: The two modes of corneal renewal. Vertical turnover is driven by progenitor cells located in the basal epithelium (left). LESC fuel centripetal renewal (right), where stem cells are in the limbus and differentiated cells localize more centrally.

5.2.1. Aging slows corneal renewal

I repeated the *Bmi1* lineage tracing experiment with another set of animals. I induced labelling at the age of 24 weeks (6 months) and collected eyeball samples 2, 4, 8, 12, 16 and 24 weeks later. To my surprise, I did not detect cell clusters nor corneal stripes in this experiment, suggesting that both vertical and limbal renewal had ceased. I assume that the rare clusters that appeared, do not suffice to maintain epithelial homeostasis. Although this is a surprising finding, it is not unexpected, as other researchers have detected a similar decrease with age as well (Dorà et al., 2015). Furthermore, the widening of limbal stripes, which is evident from chimeric and *Krt14* lineage tracing studies, suggests a decrease in the quantity of LESC clones with age (Collinson et al., 2002; Douvaras et al., 2012; Mort et al., 2009; Richardson et al., 2017). Yet, corneal tissue is maintained in older mice, but perhaps not as stably as in their young conspecifics. A study on human corneas compared the limbal morphology and colony formation efficiency between different age groups and showed that the limbus had significantly reduced area covered by the palisade structures and colony formation efficiency decreased with age (Notara et al., 2013). In line with this, corneal stability decreases with age and increases the risk of DED.

5.3. *Eda* is dispensable during LG development, but instrumental in maturation (study III)

Eda signalling guides the development of ectodermal appendages (Häärä et al., 2012; Shirokova et al., 2013; Voutilainen et al., 2015). For that reason, it is interesting that the roles of *Eda* and its signalling pathway members, *Edar*, *Edaradd* and *NF-κB* have not been elucidated in the developing cornea or LG before. Me and Dr. Kuony visualized *Eda* signalling with *in situ* hybridization, taking advantage of the RNAscope method. *Eda* localized in the (extraorbital lobe of the) LG mesenchyme from E16 to E18, however *Edar*⁺ cells positioned mostly in the epithelium of the LG ducts and acini. Rare *Edar*⁺ cells were detected in the mesenchyme. Interestingly, Pispa and co-workers showed *Edar* expression already at E14 in the developing conjunctiva and LG, suggesting that the competence to respond to *Eda* signalling arises early in LG development (Pispa et al., 2003). The pattern of EDA-EDAR localization in LG is similar to the other developing ectodermal glands, such as salivary and mammary gland, so that EDA is in the mesenchyme, whereas EDAR in the epithelium of these tissues (Lindfors et al., 2013). We confirmed that *Eda* signalling was active by using a reporter mouse line, *NF-κB-gal*, where *NF-κB*-dependent regulatory elements drive *lacZ* expression (Bhakar et al., 2002). After X-Gal staining, we detected *NF-κB* activity in the LG acini and ducts, but not in the LG mesenchyme. Furthermore, *NF-κB* activity decreased with age and became undetectable by 13 weeks of age. These results suggest a mesenchymal source of the ligand, but the effect in the epithelium during LG development.

After detection of the *Eda* signalling pathway members in the developing LG and an age-dependent decline, we hypothesized that *Eda* plays a role during LG development and that role diminishes during the postnatal maturation of the LG. We set out to analyse that putative role using a loss-of-function *Eda* mouse model, also known as *tabby* (Falconer, 1952; Srivastava et al., 1997). In this model, the *Eda* gene lacks exon 1, thus preventing the transcription of functional mRNA (Srivastava et al., 1997). Due to the X-linked nature of *Eda*^{-/-} mutation, it is important to keep in mind that all *Eda*^{-/-} samples are from male mice, whereas control samples present both males and females, depending on the experiment. However, there is no evidence of marked differences between male and female LGs during development and early postnatal time period. Thus, I suggest that this model and the use of controls is reliable in our experimental setup.

Next, we confirmed by lack of *NF-κB* activity that there was no *Eda* pathway activation in our *Eda*^{-/-} mice. The expression of *Edar* was undisturbed. However, regardless of prominent *Eda* activity during development and soon after birth, the studied mutant LGs looked morphologically similar to the LGs from control samples. This is surprising, because *Eda*^{-/-} induces drastic morphological changes, as described in HED, during the development of most ectodermal appendages (Mikkola and Thesleff, 2003). Our analysis suggests that this is not the case during LG development. We re-evaluated this by analysing the morphology of developing LGs in gain-of-function *Eda* mutants, K14-*Eda* and K14-*Edar* (unpublished data). However, these did not show altered morphology either. In hair development, the *Troy* pathway compensates for the missing EDA activity (Pispa et al., 2008). Possible redundancies between pathways in LG development remain to be elucidated.

The importance of *Eda* in LG became evident during postnatal maturation. We analysed the expression of markers of LG function and terminal differentiation from early postnatal samples, P0 to adulthood (13 weeks) with quantitative PCR (polymerase chain reaction). The selected genes display functional properties of the adult LG and are thus useful to assess the activities of the LGs

(Farmer et al., 2017). We assessed each LG compartment separately. First, in the acinar compartment the genes *Ltf*, *Bhlha15* and *Aqp5* were upregulated in *Eda*^{-/-} LGs compared to control LGs. Of these genes, *Ltf* is a major component of basal tears. Lack of *Ltf* in the tear fluid can result in microbial stress in the ocular surface and DED (Flanagan and Willcox, 2009). Furthermore, both *Ltf* and *Aqp5* guide the terminal differentiation of the LG (Flanagan and Willcox, 2009; Hirayama et al., 2017). Second, in the ductal compartment gene expression changes varied between ages. *Slc12A2* and *Krt17* were first upregulated (P0), then downregulated (P7 or P13) and again upregulated at P21. Lastly, in the myoepithelial cells *Acta2* and *Trp63* were downregulated. Evidently, *Eda*^{-/-} mutation led to misregulation in gene expression and this might change the composition of the tear fluid.

As the chosen genes reflected the functional status of the LG, we analyzed more in depth the gene expression status of the adult LGs. Thus, we employed RNA-sequencing of 13 weeks old *Eda*^{-/-} and control LGs. 1.8 % of the transcripts were differentially expressed between *Eda*^{-/-} and control samples. Of these, 1 % was missing from the mutants, whereas the 0.8 % was enriched in them. As examples, we highlighted the expression levels of a few important indicators of LG function. EGF is the main component of the basal tears produced by the LG (Klenkler et al., 2007). Loss of EDA did not affect the expression of *Egf*, however it did decrease the amount of another tear fluid component, *Cxcl10*. Interestingly, the amount of CXCL10 is also decreased in DED, suggesting a molecular link between *Eda* signaling and the development of DED in the LG (Yoon et al., 2010). However, in the previous experiment we showed an increase of *Ltf* that perhaps attempts to compensate for a decrease in *Cxcl10* to prevent or delay the onset of DED. The third mRNA we chose to highlight was *Gdf5*, which was highly upregulated. GDF5 is involved in the inhibition of corneal cell proliferation (You et al., 1999). In fact, another group has indicated a decrease in corneal epithelial cell proliferation with *Eda*^{-/-} mutation, however we could not verify this in our analysis (unpublished data) (Li et al., 2017b). Regardless, the decrease of *Gdf5* suggests that corneal stability might be compromised in *Eda*^{-/-} mutant mice. However, at this point we could not connect the transcriptomic changes to the functioning of the LG.

To understand the possible causes of these gene expression changes, we studied several indicators of LG functionality. We measured the amount of basal tears from the ocular surface using menthol and capillary methods to induce tearing and estimate tear volume, respectively (Hirayama et al., 2013). This test showed that *Eda*^{-/-} mice had a very thin tear film. In line or perhaps as a result, the blinking rate of the mutant mice was much higher than that of the control mice. Lack of meibomian glands in *Eda*^{-/-} mice could explain the increased blinking rate, however this does not explain the lower volume of tears (Wang et al., 2016). We suggest that the lower tear volume and increased blinking rate result from a defect in molecular maintenance of the LG. Our study provides evidence that *Eda* signaling is necessary to compose the basal tears.

Eda maintains the functional expression profile of the LG, but how about the cornea? In order to separate the effect of EDA in LG and cornea, we analyzed the expression pattern of *Eda* and its pathway members in the murine cornea. *Eda* is not expressed in the cornea, whereas *Edar* is. As a result, the *Eda* signaling pathway is not active. This suggests that when the cornea is affected by HED, the defects arise from dysfunctional ocular accessory organs. Interestingly, *Eda* signaling has not been studied in the conjunctiva of adult mice. In *Eda*^{-/-} mice, there were no significant defects regarding development or postnatal maturation of the cornea. However, in very old mice (more than one year old), the epithelium was thinner. Other studies have shown that corneal stability is compromised in *Eda*^{-/-} due to loss of tight junction integrity and decrease in corneal epithelial cell

proliferation (Li et al., 2017b; Li et al., 2018a). In conclusion, we showed that EDA affects tear composition and therefore contributes to the generation of DED, however, the exact mechanisms of DED pathogenesis in this case remains to be elucidated in future studies. Also, it is good to keep in mind that DED is a multifactorial disease, where other components of the ocular surface; cornea, conjunctiva and meibomian glands, are also involved in the pathophysiology.

5.4. Corneal wounding by abrasion is a useful tool to study healing and the cornea-LG connection (studies I, II and III)

The main part of this research work has been the development and use of corneal abrasion to induce a corneal wound. This method has been in use in corneal research for very long, however a detailed protocol describing it did not exist. This led to the situation where each research group generated their individual approach to the abrasion method and this challenges the interpretation and comparison of results between groups.

Abrasion, or debridement, of the corneal epithelium was initially developed for *ex vivo* cornea using dulled scalpels or blades (Gipson and Kiorpes, 1982). This method has later been used *in vivo* on mouse, rat, and rabbit (Danjo and Gipson, 1998; Lyu and Joo, 2005; Nagata et al., 2015; Pal-Ghosh et al., 2008b; Stepp and Zhu, 1997; Stepp et al., 1996). These studies were important to optimize medical treatments of ocular surface diseases. An ocular burr replaced the scalpels and blades, because the burr is user friendly and hardly ruptures the cornea; the tip of the burr is dulled or round. The most frequently used ocular burr, AlgerbrushII, is used by the ophthalmologists to remove a particle from the ocular surface or clear irregularities off of wound edges (Stepp et al., 2014).

In this method, I anesthetized adult mice prior to the operation and then kept them on a heat plate (+37°C) for the duration of the operation. To perform the abrasion, I opened the eyelids and kept the eye exposed. I used the rotating option (vibration) of the burr and moved the burr tip gently, but firmly back and forth on the cornea a few times. I followed abrasion immediately with fluorescein staining and imaging of the cornea to visualize the extent of the abrasion. Of note, fluorescein ($C_{20}H_{10}O_5Na_2$) is a chemical compound that is used by ophthalmologists to assess aspects of tearing such as tear break-up time as well as corneal and conjunctival surface damage. Fluorescein staining is necessary to follow the wound healing timeline of the cornea. Because the abrasion is made by hand, the shape and size of the abraded region always varies slightly. This might affect wound closure timeline. However, once the method is well established, the abrasion will appear quite similar each time. After imaging is finished, I placed a drop of antimicrobial ointment on the ocular surface and woke up the animal. This setup provides a quick and easy method for experimental abrasion on the cornea. In addition, the abrasion could be targeted to any region of the cornea. This type of abrasion was used to remove limbal epithelium and study LSCD and the healing capacity of non-stem cells of the murine cornea (Nasser et al., 2018; Richardson et al., 2018). Furthermore, this method allows for different shapes and sizes of the abrasion.

The most critical point of the abrasion method is the scraping of the cornea. Here, the choice of a correct tool is pivotal. Most reports claim that the ocular burr does not harm the BM, but only removes corneal epithelium, thus inducing a “non-penetrating” injury (Boote et al., 2012). Other researchers compared a dulled blade and the ocular burr and showed that the burr does penetrate the BM, however at wound margins the BM remains intact (Pal-Ghosh et al., 2011). Interestingly, in rat

the entire BM remained untouched, perhaps because the cornea is thicker in rat than in mouse (Pal-Ghosh et al., 2011). This is of importance, because the presence of BM can support re-epithelialization after abrasion. Unfortunately, we did not evaluate the presence of BM markers using our method. The presence or absence of the BM after wounding can be evaluated with antibodies against laminin 332 and/or type VII collagen on histological sections (Stepp et al., 2014). Also, factors such as applied force, time of abrading and vibration speed affect the penetrance of the burr through the BM. In conclusion, both dulled blade and ocular burr offer a suitable option for non-penetrating injuries, but the possible damage in BM should be evaluated for proper interpretation of the results.

There is a multitude of different types of corneal injuries and the mechanism of healing depends on the size, depth and cause of the trauma. In this model and other studies, the wound closed by the third day (72 hours) after abrasion (Denaxa et al., 2009). At 18 hours, the wound size was already diminished. I, and others, showed that this occurs via re-epithelialization without cell proliferation (Chung et al., 1999). In support, inhibition of cell divisions with mitomycin C in cell culture does not affect wound closure (Kaplan et al., 2012). I illustrated that the epithelium becomes thinner from a large region between the wound edge and the limbus at 18 hours post-operation, which coincides with an intense period of re-epithelialization. In support, epithelial migration rate increases upon abrasion injury (Mort et al., 2009). I showed that 18 hours after abrasion the wound margins were not sharp and defined, but there was a thin, acellular continuation of the epithelium. Others confirmed that cells at the wound edge undergo shape changes during the re-epithelialization process (Park et al., 2019). This might provide a ledge for the epithelial cells to start migration over the exposed region (Stepp et al., 2014). Others propose that the migrating epithelium moves as a sheet of connected cells, where an intracellular actin filament string and adhesions between cells and possible the substrate (BM) support the movement (Danjo and Gipson, 1998; Theveneau and Mayor, 2013). Surprisingly, mice of different strains exhibit different corneal wound healing rate due to strain-specific differences in epithelial migration (Pal-Ghosh et al., 2008a). Closure is followed by re-stratification of the corneal layers (Suzuki, 2003). During maturation, cell proliferation fuels the initial stratification of the corneal epithelium, suggesting that a similar mechanism might be in play during wound healing (Francesconi et al., 2000). Proliferative peak in the healing cornea was witnessed at 24 hours after abrasion, just after wound closure (Park et al., 2019; Sagga et al., 2018).

There is a limit to the healing potential of the cornea. Stepp and co-workers reviewed studies suggesting that if the abrasion is 1.5 mm wide or smaller, the wound closes spontaneously and corneal epithelial cells do not undergo marked morphological changes (Stepp et al., 2014). However, when the abraded region encompasses 2 mm or more of the mouse cornea, wound closure varies from days to weeks, if it closes at all (Pal-Ghosh et al., 2004; Stepp et al., 2014). Remarkably, the average diameter of the mouse eye is 3 mm, so the latter type of abrasion comprises almost the entire cornea. In the large abrasions, the remaining epithelial cells started to express the limbal marker $\alpha 9$ integrin (Pal-Ghosh et al., 2004; Stepp and Zhu, 1997). This suggests that the remaining epithelial cells adopt an undifferentiated state to re-establish the corneal epithelium. This hypothesis gains support from a studies where a large (2-2.5 mm) corneal abrasion or trephine injury resulted in temporary downregulation of *Krt12* and upregulation of limbal markers *Krt18*, *Krt19*, *Krt14* and *Krt15* (Nowell et al., 2016; Pajoohesh-Ganji et al., 2016; Park et al., 2019). This change in keratin expression pattern lasted until 42 days after the abrasion indicating that the process of healing undergoes in a molecular level way past wound closure. I detected *Krt14* expression throughout the epithelium after abrasion (unpublished data), suggesting that this model induces a “large” wound. This shows that the choice

of wound size is crucial as the epithelial cells undergo a different type of healing process based on wound size. The abrasion did not induce adverse pathologies, such as inflammation, neovascularization, or opacification of the cornea.

5.4.1. *Bmi1*+ corneal epithelial progenitor cells do not contribute to corneal wound healing

What is the role of corneal epithelial progenitor and stem cells in the process of wound healing? After assessing the basic mechanisms of wound healing, I used the *Bmi1* lineage tracing approach to analyze the role of the *Bmi1*+ progenitor cells after abrasion injury. For this experiment, I induced *lacZ* expression at the age of 6 weeks and followed this with abrasion at 8 weeks of age. I collected the cornea samples during the re-epithelialization period; 18 and 72 hours after abrasion. Fluorescein staining revealed that the abrasion closed normally. However, *Bmi1* lineage tracing showed that the epithelial cell turnover by the *Bmi1*+ progenitors remained unchanged during wound closure. Similarly, as in uninjured corneas, the *Bmi1*+ cell clusters appeared in central, peripheral and limbal epithelium, except the abraded area. This suggests that the *Bmi1*+ cells do not respond to an injury but maintain the homeostatic renewal.

Because *Bmi1* is not expressed in the LESC, this experiment did not display possible limbal activation as a result of the abrasion. A recent lineage tracing on *Krt14*+ cells proposed that epithelial abrasion increases limbal renewal and forces wound closure by basal cell migration (Park et al., 2019). However, other reports claim that the cornea can recover without limbal input (Chang et al., 2008; Majo et al., 2008). This is supported by evidence from patients with LSCD, who maintain clear islets on the cornea over 5 years (Dua et al., 2005). Furthermore, peripheral corneal cells can re-establish the LESC after limbally targeted abrasion (Nasser et al., 2018). These results suggest that both central and limbal epithelial cells have the capacity to respond to ocular surface injury, possibly exerting different roles in the process of wound healing.

5.4.2. Corneal abrasion induces gene expression changes in the LG

As the final step of this research work, me and Dr. Kuony wanted to study the role of LG in corneal wound healing. Growth factors secreted by the LG enhance healing after corneal insult (Klenkler et al., 2007). We analyzed the LG gene expression of major growth factors, *Tgfb1* and *Hgf*, 18 hours after abrading the cornea. These factors were selected because they are present in prominent concentrations in human tears and likely originate from the LG (Li et al., 1996; Yoshino et al., 1996b). *Fgf7* is a novel factor that has not been identified in the tear fluid before. We determined that the expression of *Tgfb1* was upregulated, whereas *Fgf7* was downregulated in the LG after abrasion. *Hgf* remained unchanged. These results show that the cornea and LG have an intriguing connection as the damage to cornea induces changes to LG function. A similar phenomenon is common in salivary and sweat glands, where their fluid secretion changes upon oral or skin wounds, respectively (Bodner, 1991; Rittié et al., 2013). Thus, cornea and LG maintain a constant conversation to regulate cellular behaviors in homeostasis and disease.

Our following experiments focused on *Eda* signaling during wound healing, because loss-of-function *Eda* mutation was shown to delay corneal wound healing and because EDA protein is a component of the tear fluid (Li et al., 2017a). However, the mentioned work concluded that EDA in

the tears is secreted by the meibomian glands. We followed wound healing with fluorescein staining in *Eda*^{-/-} mice after abrasion and confirmed that the healing process is slower in these mice. There was still a large exposed area in the *Eda*^{-/-} cornea 18 hours after the damage was inflicted. Next, we analyzed if changes in LG function could explain this delay, because there was no difference in *Eda* signaling status in the cornea suggesting that the delay arises from a non-corneal origin. Indeed, after corneal abrasion, *Eda* and *Edar* expression and NF- κ B activity decreased drastically in the LGs. Thus, downregulation of *Eda* signaling is necessary to produce the reflex tears after corneal injury.

When we analyzed the LGs of *Eda*^{-/-} mice after corneal abrasion, we again saw a change in the expression levels of secreted growth factors *Tgfb1* and *Fgf7*. Here, also *Hgf* level decreased. These changes reflected the changes in normal LGs after abrasion but were more modest. In addition, the result indicated that the general expression level of the growth factors was lower in *Eda*^{-/-} LG than in normal conditions. We were unable to assay EDA and the growth factors in the lacrimal secretion, so it remains to be seen whether gene expression change implies that the LG modulates tear fluid contents in corneal injury. Thus, this work does not exclude the possibility that corneal defects in *Eda*^{-/-} animals originate from the meibomian glands, however we showed that the decrease in LG tear volume and blinking rate contributes to the *Eda*-associated DED. Thus, these experiments provides an important insight into LG biology in *Eda* mutation.

To our great surprise, we detected a bilateral effect in the LGs after a unilateral abrasion. Here, corneal abrasion induced an upregulation of *Tgfb1* and downregulation of *Fgf7* in the bilateral cornea that was not abraded. We evaluated the growth factor expression levels in the bilateral cornea of the *Eda*^{-/-} mice and detected the same change in *Tgfb1* and *Fgf7* expression. However, *Hgf* level was downregulated in the bilateral cornea, similarly to the abraded eye. These results are particularly interesting, because they suggest that both LGs respond similarly to corneal abrasion. Recently, another group showed similar bilateral effect on cornea and LG after corneal nerve cutting (Lee et al., 2019). There might be an anatomical explanation to this issue. Corneal afferent and LG efferent nerves interconnect in the trigeminal ganglion, but perhaps there is no segregation between the two branches of LG nerves at this level (Meng and Kurose, 2013). Thus, both LGs receive the same signal to switch from basal to reflex tear secretion. Lee and colleagues showed also that inducing a keratectomy injury on the cornea initiated an immune response (Lee et al., 2019). Systemic immune signaling could provide another route for communication between the cornea and the LG. In severe cases, unilateral injury might result in the development of bilateral corneal responses, if stromal cells or conjunctival cells respond by inducing neovascularization or conjunctivalization, respectively. In these cases, originally unilateral injury leads to blindness of both eyes.

6. Concluding remarks and future perspectives

Balance between stem cell self-renewal and differentiation is necessary to maintain homeostasis. In order to achieve this, different cellular compartments of the tissue, the stem cells, progenitors and differentiated cells must communicate with each other. In the cornea, LESC's reside in the limbus at the corneal periphery (Cotsarelis et al., 1990; Lehrer et al., 1998). The immediate progeny of the stem cells, the early progenitor cells, are also located in the limbal basal layer (Lehrer et al., 1998). Many consider these early progenitors phenotypically and functionally indistinguishable from the stem cells. With each division, the progenitors localize further away from the limbal stem cell niche and lose their proliferative capacity. I identified the expression of *Bmi1* in the corneal, epithelial progenitors. Importantly, I detected these progenitors in all corneal regions, limbal, peripheral and central cornea. I also dissected the epithelial turnover time by using *Bmi1* lineage tracing and showed that the corneal epithelium is vertically renewed in 2-8 weeks. Other markers of corneal, epithelial progenitors have been suggested, however not studied with lineage tracing (Bhattacharya et al., 2019; Menzel-Severing et al., 2018). It is possible that several progenitor and stem cell subpopulations with different dynamics inhabit distinct regions of the cornea, similarly to other tissues such as hair and intestine (Greco and Guo, 2010; Rompolas et al., 2013). The exact expression domains and dynamics of these cells remain to be elucidated.

Our work sheds light on the biology of *Eda* signalling in the cornea and LG. Interestingly, even though the role of *Eda* is well characterized in a multitude of ectodermal appendages, there were only few studies in relation to the eye. These concentrated on adult cornea and meibomian glands, but not on LGs (Li et al., 2017a; Li et al., 2018b; Wang et al., 2016). Even though *Eda* seemed to be dispensable during LG development, the expression in postnatal LG could fine-tune the maturation of the gland to gain proper functionality. Together with Dr. Kuony, we showed that *Eda*^{-/-} mutation resulted in DED, indicated by decreased tear volume and increased blinking rate. These results highlight that the LG is an important target to treat DED.

Currently, DED can be treated with lubricating eye drops with or without added medical agents. However, if the DED is caused by X-linked HED, i.e. loss-of-function mutation in *Eda*, the patient is likely to suffer from a multitude of other symptoms as well. Unfortunately, a clinical trial on human patients with EDI200 (containing EDA recombinant protein) to treat this condition was not successful. This leaves clinicians to treat the variable symptoms individually. To target the LG for treatment of X-linked HED, it is possible to utilize an injection model, where the protein (for example EDA) under testing, is placed directly inside the LG (Zoukhri et al., 2007). EDA addition to cornea organ culture medium partly rescued the *Eda*^{-/-} phenotype in the cornea (Li et al., 2017a). This is promising, as eye drops with EDA could provide an avenue for treatment of *Eda*-linked DED and might even help with the other symptoms of HED in human patients due to the leakage of medical substances to the systemic circulation (Uusitalo and Salminen, 1998).

Lastly, I dissected the roles of *Bmi1* and *Eda* in corneal wound healing. Using *Bmi1*, I showed that after abrasion the corneal epithelium follows regular turnover and the wound closure occurs via cell rearrangement and migration rather than proliferation. In addition, the expression of *Eda* is decreased in the LG after abrasion. I propose a model, where *Eda* decrease is essential to the production of reflex tears. These reflex tears are a critical component of the healing cornea (Klenkler et al., 2007). The logical next step would be to analyse the protein composition of mouse tears before and after corneal abrasion.

Understanding the cellular and molecular steps of corneal wound healing present an avenue for better treatment of ocular surface trauma. Many patients suffer from ocular surface disease from poorly healed superficial or more severe corneal damage. Some factors, such as EGF and a placenta-derived peptide JBP485, were shown to both promote corneal wound healing and also surpass the required safety level for medical use (Kitazawa et al., 1990; Nagata et al., 2015). The logical route is via eye drops. This thesis suggests that understanding the cornea-LG feedback loop in homeostasis and pathology provides tools to treat the various diseases of the ocular surface.

I studied corneal wound healing until the epithelium was closed at 72 hours. Even though this type of superficial wound re-epithelializes quickly, the process of healing is still ongoing for days after closure. This was shown by collective and temporal change in the expression of corneal keratins and *Mmp12*, the latter localizes to the ECM (Pajooohesh-Ganji et al., 2016; Park et al., 2019; Wolf et al., 2017). My unpublished work supports these findings. Further studies are necessary, because understanding the physiological meaning of these gene expression changes lies in the centre of the healing process. In addition, I noticed a change in gene expression also in the bilateral, unwounded cornea. This phenomenon is of interest to us. This thesis provided the important groundwork and opened new research questions for future studies on corneal wound healing. The research continues.

In this thesis, we have demonstrated that (1) *Bmi1* is expressed in the corneal, epithelial progenitor cells, (2) but do not contribute to corneal wound healing. (3) *Eda* contributes to functional maturation of the LG. (4) Lack of EDA leads to dry eye disease and (5) *Eda* signalling is inhibited during corneal wound healing suggesting that *Eda* participates in the maintenance of a stable corneal barrier.

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